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GRANT NUMBER: DAMD17-94-J-4291

TITLE: Cell-Matrix Interactions in Breast Cancer Cells

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REPORT DATE: September 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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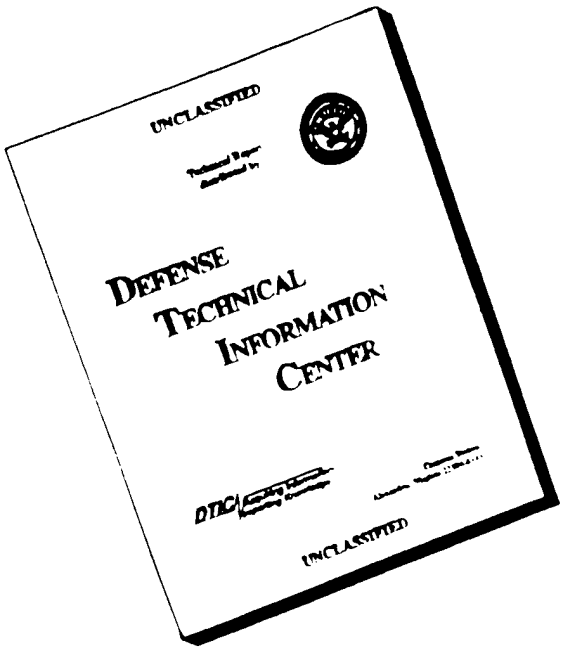

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1996	Annual (15 Aug 95 - 14 Aug 96)

4. TITLE AND SUBTITLE
Cell-Matrix Interactions in Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-94-J-4291

6. AUTHOR(S)
Jonathon C. Jones, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Northwestern University
Evanston, Illinois 60208-1110

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

During the report period we have shown that loss of expression of the hemidesmosome associated matrix component laminin-5 in breast epithelial tumors is correlated with an apparent phosphorylation of the alpha 6 integrin, a subunit of a laminin-5 receptor. Thus phospho-epitope specific alpha 6 integrin antibodies have potential use in diagnosis of malignant breast cells. In addition, we have developed a model of breast epithelial morphogenesis using MCF-10A cells and have analyzed the potential role of hemidesmosome components in morphogenetic events.

14. SUBJECT TERMS Breast Cancer
Hemidesmosomes, matrix, matrix receptors, morphogenesis

15. NUMBER OF PAGES 77

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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JCAdams 8/16/96

PI - Signature Date

(5) Introduction

Modulation in adhesion of tumor cells compared with their normal counterparts plays an important role in development of invasive potential. Our application, as funded, concerns the fate of certain epithelial cell-matrix connectors called hemidesmosomes which are involved in anchoring breast epithelial cells to underlying matrix (1,2,3). Loss of hemidesmosomes appears characteristic of more invasive epithelial tumor cells. Yet there have been very few studies on hemidesmosomes in either normal or tumor breast epithelial tissues (2). This is because of limited availability of cell and molecular probes for individual hemidesmosome components until recently (1,3). It is the goal of the studies that were detailed in our original application to analyze hemidesmosomes and hemidesmosomal components in a variety of breast tumor types. One major aspect of our work is to identify hemidesmosome-related markers which could be used in diagnostics. Our approach combines cell and molecular biological analyses of normal and tumor tissue as well as normal and tumor cells maintained in vitro.

(6) Body

The following were the set of tasks funded by the US Army in 1994. These are a revision of those tasks originally proposed in the Fall of 1993 and were approved by the US Army prior to funding.

Task 1. Immunochemical characterization of hemidesmosome components in normal and tumor breast tissue and their cultured cell counterparts
Task 2. Molecular analyses where message levels of HD proteins will be determined (complimentary to task 1).
Task 3. Electron microscopic evaluation of normal and tumor material
Task 4. Cell assays in matrigel

In the first year of the award we undertook studies revolving around tasks 1 and 3 i.e. expression of hemidesmosome components in normal and diseased breast tissue as well as normal and tumor cells maintained in vitro. These studies were published in Bergstraesser et al. (1995) which is enclosed as part of the Appendix. In addition, in the latter publication we described the ultrastructure of hemidesmosomes in normal tissue and detailed the ultrastructure of tumor cells in situ. These investigations were outlined in last year's Progress Report and are available for critique in a peer reviewed journal article (Bergstraesser et al., 1995). Thus, to prevent redundancy, they will not be related again here. However, over the last year we have extended these observations. We have also made progress with regard to Task 4. These results are detailed below, together with our aims for the next grant period.

Task 1

One of our motivations in carrying out the studies outlined under task 1 was to identify potential new diagnostics for invasive breast cancer cells. In our search for such reagents, we have begun to extend the observations in Bergstraesser et al. (1995)

by investigating hemidesmosome integrin and hemidesmosome matrix components in normal and tumor breast tissue, with particular regard to laminin-5, a newly identified hemidesmosome component of basement membrane, and a receptor for laminin-5, namely the hemidesmosome integrin, $\alpha 6\beta 4$ (Jones et al., 1994). Some of our studies were premised on experiments in the laboratory which indicate that epithelial cell interaction with laminin-5 triggers a dephosphorylation of the $\alpha 6$ integrin subunit.

MATERIALS AND METHODS

Cell Culture

804G cells were maintained in Dulbecco's Modified Eagles' Medium (DMEM) (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum, penicillin (100 I.U./ml) and streptomycin (100 mg/ml). Conditioned medium was obtained from 804G cells one day post-confluency. FG cells were plated directly into 804G conditioned medium, or DMEM with 10% fetal calf serum, penicillin (100 I.U./ml) and streptomycin (100 mg/ml).

Antibodies

Monoclonal antibody GoH3 against the $\alpha 6$ integrin subunit was purchased from ImmunoTech (Westbrook, ME). The polyclonal antibody 6844, against the cytoplasmic domain of the integrin subunit $\alpha 6A$, were kind gifts from Dr. Vito Quaranta (Tamura et al., 1990). The mouse IgG1 monoclonal antibody 4E9G8 against $\alpha 6$ integrin subunit was purchased from ImmunoTech (Westbrook, ME). GB3, a mouse monoclonal antibody which recognizes the γ chain of human laminin-5, was obtained from Harlan Bioproducts for Science, Inc. (Indianapolis, IN) (Verrando et al., 1987, Matsui et al., 1995).

Tissue

Breast tissues were obtained from Northwestern Memorial Hospital, Evanston Hospital, or the Cooperative Human Tissue Network of the National Cancer Institute. All malignant tissues used were infiltrating ductal carcinoma varying from grade I to III, and were from patients with from 0 positive lymph nodes to 10/10 positive level III nodes. Normal breast tissue was derived from reduction mammoplasties.

Immunofluorescence

Sections of frozen breast tissue were prepared on a Tissue-Tek cryostat and placed on glass slides. Cells grown on coverslips and tissue sections on slides were processed for immunofluorescence as described in Klatte et al. (1989) and Riddelle et al. (1992). Appropriate secondary antibodies were purchased from Southern Biotechnology (Birmingham, AL). For mouse/rat double labels, goat anti-mouse fluorescein antibodies pre-adsorbed against rat IgG and goat anti-rat rhodamine antibodies pre-adsorbed against mouse IgG were purchased from Southern Biotechnology. Coverslips or slides were viewed on an LSM10 confocal microscope or a Photomicroscope III (Carl Zeiss, Thornwood, NY). Unless stated, all images

were taken within 0.5μm of the substrate attached surface of the cell. No adjustment of focus was made when changing lasers to analyze double labels. Images were stored on Sony magneto-optical disks (Inmac, Irving, TX) and printed on a Tek Phaser IISDX color printer (Tektronics, Beaverton, OR). For all immunofluorescence studies we use non-immune IgG as controls. This allows us to assess non-specific binding of fluorochrome conjugated secondary antibodies.

Immunoprecipitation

Cells were solubilized in Tris-buffered saline (TBS, pH 7.4) containing 0.5% NP-40, 2 mM CaCl, 1mM PMSF, 100 mM leupeptin, 1 mM pepstatin and 1 μg/ml aprotinin. After clarifying at 15,000 rpm for 15 mins at 4^0C, the supernatant was collected and precleared with protein-G agarose beads (GIBCO). The supernatant was then rotated at 4^0C with antibodies for 2 hr and then protein-G agarose beads were added for an additional 2 hr. The samples were centrifuged, the supernatant discarded, and the beads washed 3 times in a wash buffer (TBS containing 0.5% NP-40). After the final wash, the beads were boiled in Laemmli sample buffer (Laemmli, 1974) containing 10% β-mercaptoethanol (βME) for 5 mins.

For ^{32}P radiolabeling, subconfluent cultures of FG cells were washed and then incubated in phosphate free medium for 30 mins at 37^0C. Next, cells were passaged and replated into 60mm dishes in the presence or absence of laminin-5 in phosphate free medium. Immediately after plating, 0.1mCi/ml of [^{32}P] orthophosphate was added to each dish. After radiolabeling, cells were rinsed in TBS and subjected to immunoprecipitation as detailed above using GoH3 antibody. To inhibit phosphatase and kinase activity, 10mM sodium flouride, 4mM sodium orthovanadate, 10mM sodium pyrophosphate and 4mM EDTA were added to the immunoprecipitation lysis and wash buffers. Samples were analyzed by SDS-PAGE on 12% gels. Gels were either dried and exposed to film or separated proteins were transferred to nitrocellulose which was then processed for immunoblotting with 6844 α6A polyclonal antibodies. Autoradiographs and immunoblots were scanned and quantitated using Intellegent Quantifier (Bio Image, Ann Arbor, MI).

Peptide preparation

The peptide, NH_2-CIHAQPSDKERLTSDA-COOH, and phosphopeptide, NH_2-CIHAQP-pS-DKERLTSDA-COOH, were prepared commercially (Research Genetics, Huntsville, AL). These peptides match the amino acid sequence (residues 1036 to 1050) of a portion of the cytoplasmic domain of the α6A integrin subunit (Tamura et al., 1990). The amino terminal cysteine residue was included for future conjugation purposes. The phosphorylated serine in the second peptide corresponds to serine residue 1041 which has been shown to be phosphorylated in vivo (Hogervorst et al., 1993). Peptides were spotted onto PVDF membrane (Bio-Rad, Richmond, CA) which was subsequently processed for immunoblotting as detailed below.

SDS-PAGE and immunoblotting

Whole cell extracts were made by solubilization of a confluent 100 mm dish of cells in 1 ml of Laemmli type sample buffer containing 10% βME (Laemmli, 1974). SDS-PAGE and immunoblotting were carried out as described previously (Zackroff et al., 1984; Klatte et al., 1989). Laminin-5 was purified from 804G conditioned medium as previously detailed (Baker et al., 1996). Approximately, 1.0 μg bovine serum albumin (BSA) was added to 5.0 μg of laminin-5 as a protein stabilizer.

RESULTS

Laminin-5 and the Phosphorylation State of α6 Integrin

Our interest in the use of antibodies specific for phosphoepitopes on α6 integrin was triggered by studies where we investigated the impact of culturing cells of the FG pancreatic carcinoma line in the presence of laminin-5 on the physiological state of α6 integrin. When FG cells are plated into laminin-5 rich medium, they "capture" laminin-5 and incorporate it into their matrix in an arc or circle type organization (Baker et al., 1996a).

For our studies we made use of monoclonal antibody 4E9G8 which has been reported to be specific for a phosphorylation sensitive epitope on α6A integrin subunit, the predominating splice variant expressed in FG cells, and rat monoclonal antibody GoH3 which recognizes the α6 integrin subunit irrespective of its phosphorylation state (Hogervorst et al., 1993). GoH3 antibodies stain FG cells, maintained under their normal culture conditions, in a streaky pattern towards their substratum attached surface (Fig. 1). In contrast, 4E9G8 antibodies fail to recognize comparable FG cells as determined both by immunoblotting and immunofluorescence (Figs. 2A,3A). Conversely, in FG cells incubated in laminin-5 containing medium, 4E9G8 antibody shows an arc or circle of staining towards the cell-substratum interface (Fig. 3C). This is the same pattern of organization seen using laminin-5 antibodies (Baker et al., 1996a). These arcs or circles co-localize with staining generated by GoH3 antibodies (Fig. 3E,F). In addition, 4E9G8 antibody reacts with the α6A "light" chain, migrating at 25kD, in extracts of FG co-incubated in laminin-5 containing medium (Fig. 2A).

To further define the epitope of 4E9G8 antibody, we generated peptides consisting of residues 1036-1050 of the α6A integrin subunit cytoplasmic domain. In one of our peptides, serine residue 1041 was phosphorylated. This peptide was not recognized by 4E9G8 antibodies unlike its non-phosphorylated counterpart (Fig. 2B). Both phosphorylated and non-phosphorylated peptides are recognized by a rabbit serum 6844 directed against the α6A integrin cytoplasmic domain (Fig. 2B).

To gain independent confirmation that the α6 integrin undergoes a dephosphorylation in FG cells maintained in the presence of laminin-5, we undertook an in vivo phosphorylation assay (Fig. 2C). The α6 integrin was immunoprecipitated using GoH3 antibodies from ^{32}P radiolabeled FG cells, maintained either in the absence or presence of laminin-5. Equal amounts of α6 integrin, precipitated from FG cells maintained under the two distinct conditions, were loaded onto gels as

shown by the immunoblot using serum 6844. There is an apparent 37% decrease in the level of phosphorylation of the 25kD α6 integrin light chain in cells maintained in the presence of laminin-5 when compared with control cell immunoprecipitates (Fig. 2C).

Analyses of breast tissues for expression of laminin-5 and α6 integrin

The above studies have now led us to begin to study the phosphorylation state of α6 integrin in normal and tumor breast tissues. For our studies, normal breast tissues were obtained from reduction mammoplasties and invasive tumor breast tissue biopsies were surplus to pathological examination. Tissues were processed for double label immunofluorescence microscopy using a rat monoclonal antibody against a6 integrin (GoH3), which recognizes the α6 integrin subunit irrespective of its phosphorylation state, in combination with either laminin-5 antibodies (GB3) or antibody 4E9G8 against a phospho-epitope of α6 integrin. In normal breast tissue, 4E9G8 antibodies stain sites of epithelial cell/matrix interaction and co-localize with laminin-5 antibodies (Fig. 4). Similarly, GoH3 antibodies co-localize with GB3 antibodies (Fig. 4). In contrast, in invasive tumors, there is a loss of expression of laminin-5 at sites of tumor cell/matrix association and this correlates with a loss of staining by 4E9G8 antibodies but not by GoH3 antibodies (Fig. 4).

SUMMARY

Our results indicate that there is a loss of laminin-5 in the matrix of invasive breast tumor cells. This is consistent with a loss of hemidesmosomes in such cells as determined by electron microscopy (Bergstraesser et al., 1995). In addition, loss of laminin-5 expression by tumor cells correlates with a dephosphorylation of the α6 integrin subunit.

We have only analyzed five invasive tumor specimens to date in the above manner. Nonetheless, our results suggest the exciting possibility that we can use a combination of the GoH3 and 4E9G8 antibodies to specifically mark invasive tumor cells i.e. cells which express phosphorylated α6 integrin may be more invasive. However, to validate this possibility, we now need to study non-invasive breast tumors as well as many more invasive tumor specimens. This is one of the goals of the next grant period.

Task 4

Extracellular matrix plays a crucial role in determining the morphogenesis of a number of epithelial tissue types (Hay, 1993). One of the most dramatic examples of this phenomenon is the regulation of mammary epithelium phenotype by elements of basement membranes derived from the Engelbreth-Holm-Swarm tumor ("matrigel")(Bissell and Ram, 1989; Barcellos-Hoff et al., 1989; Blum et al., 1989; Lin and Bissell, 1993). Indeed, mouse mammary epithelial cells assemble into structures remarkably similar to alveoli of lactating mammary glands and produce milk proteins when maintained in matrigel (reviewed in Lin and Bissell, 1993).

Compared with the rodent system, analyses of morphogenesis of human mammary epithelial cells has progressed more slowly, in part because of difficulties in maintaining cultures of primary human cells. This problem has been partially alleviated by the development of media for the culture of primary human mammary epithelial (HMEC) cells although establishment of primary cultures remains problematic (Stampfer, 1985; Bergstraesser and Weitzman, 1993). One alternative is the use of continuous human mammary epithelial cell lines such as MCF-10A (Soule et al., 1990). Indeed, a model for the study of mammary epithelial cell morphogenesis using MCF-10A cells has recently been described (Howlett et al., 1995).

It has now been shown that laminin-1 is the matrix component of matrigel which regulates morphogenesis as well as milk protein expression of mouse mammary epithelial cells in vitro (Streuli et al., 1995). Furthermore, the domain responsible for such regulation resides in the so-called E3 fragment of laminin-1 and is located towards the carboxy terminus of the α1 subunit of the heterotrimer (Streuli et al., 1995). Laminin-1, via its cell surface receptors, is believed to establish polarity of mammary epithelial cells, a process which is an essential prerequisite to cell differentiation (Streuli et al., 1995). However, following polarization, it is hypothesized that epithelial cells modulate their own microenvironment by producing additional basement membrane components (Bissell and Ram, 1989). The latter could include a number of laminins since laminin-1 is only one of several laminin isoforms which occur in intact basement membranes (Timpl and Brown, 1994). For example, laminin-5 is widely distributed in the basement membranes of epithelial tissues, including the mammary gland, as we show here (Verrando et al., 1987; Rousselle et al., 1991; Carter et al., 1991; Kallunki et al., 1992; Timpl and Brown, 1994). Do these endogenously secreted basement membrane elements play a role in mammary epithelial morphogenesis? To answer this question, we have analyzed the function of laminin-5 in an in vitro model of mammary epithelial morphogenesis using MCF-10A cells. These cells undergo branching morphogenesis i.e. assemble a highly anastomosed multicellular network, when cultured on matrigel. We show that matrigel-induced differentiation of MCF-10A cells is inhibited by function blocking laminin-5 antibodies as well as antibodies against two distinct laminin-5 receptors. Since laminin-5 is a component of certain cell-matrix junctions called hemidesmosomes and MCF-10A cells assemble hemidesmosomes in vitro, we discuss the possibility of signaling events transduced by these complex morphological entities. This work is in press in the Journal of Cell Science and a preprint is included in the Appendix (Stahl et al.).

MATERIALS AND METHODS

Cell Culture

MCF-10A cells were obtained from American Tissue Culture Collection (Rockville, MD) and were maintained in a 1:1 mix of DME and Ham's F12 media supplemented with 5% equine serum, 0.01 mg/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin, and 500 ng/ml hydrocortisone. SCC12 cells were maintained in a serum free growth medium (Medium 154)(Cascade Biologics, Inc., Portland, OR).

For our morphogenesis assays, matrigel was purchased from Collaborative Biomedical Products (Bedford, MA) and was coated onto plastic dishes at approximately 15 mg/ml. The dishes were subsequently incubated at 37^{0}C for 30 minutes prior to addition of cells. In some instances, cells were mixed with liquid matrigel at 4^{0}C. The cell/matrigel mix was then pipetted onto plastic and allowed to gel at 37^{0}C.

Antibodies

GB3, a mouse monoclonal antibody which recognizes the γ chain of human laminin-5, was obtained from Harlan Bioproducts for Science, Inc. (Indianapolis, IN) (Verrando et al., 1987, Matsui et al., 1995). Mouse monoclonal antibody, clone 17, specific for the β chain of laminin-5 was purchased from Transduction Laboratories (Lexington KY). Dr. William Carter, Fred Hutchinson Cancer Research Center generously provided C2-9, a function blocking mouse monoclonal antibody specific for the α3 chain of laminin-5 and P1E1, a non-function blocking antibody which also recognizes the α3 chain of human laminin-5 (Xia et al., 1996). We used P1E1 as a control IgG in some of our antibody inhibition studies. The rabbit serum J17, against BP180 and the mouse monoclonal antibody 10C5, against BP230, have been described elsewhere (Hopkinson et al., 1992; Hopkinson and Jones, 1994). GoH3, a rat monoclonal which recognizes the α6 integrin subunit, was purchased from Immunotech (Westbrook, ME). P1B5 and 3E1, mouse monoclonal antibodies which recognize the α3 integrin and β4 integrin subunits respectively, were purchased from Gibco BRL (Gaithersburg, MD). Rabbit sera 6945 and 6845, against β4 integrin and the "light" chain of the α6 integrin subunit respectively, were kindly provided by Dr. Vito Quaranta, Scripps Institute (Tamura et al., 1990) .

Immunofluorescence

MCF-10A cells, maintained on glass coverslips, were either permeabilized in acetone at -20^{0}C for 2 minutes and air dried thoroughly, or, for integrin localization, were first fixed for 5 minutes in 3.7% formaldehyde, washed thoroughly in PBS, and then permeabilized in acetone at -20^{0}C for 2 minutes prior to air drying. Cells maintained in matrigel were prepared for immunofluorescence analyses by first fixing them for 10 minutes in 3.7% formaldehyde. After washing thoroughly in PBS, they were permeabilized with 0.5% Triton-X 100 in PBS at 4^{0}C for 10 minutes and then washed once again in PBS. Preparations were incubated with primary antibody diluted in PBS at 37^{0}C in a humid chamber for 1 hour, washed 3 times in PBS, and

incubated with an appropriate fluorochrome conjugated secondary antibody for a further 1 hour at 37^{0}C.

For frozen tissue sections, normal human breast tissue from a reduction mammoplasty was received from the Cooperative Human Tissue Network (Columbus, OH). Tissue was snap frozen in liquid nitrogen and embedded in Tissue-Tek OCT compound (Miles Laboratory, Elkhart, IN). 10 μm thick sections of the frozen tissue were prepared and mounted on poly-L-lysine coated microscope slides. Sections were fixed for 5 minutes in -20C acetone, air-dried thoroughly, and stained for immunofluorescence as above.

Fluorescence specimens were visualized using a Zeiss LSM10 laser scanning confocal microscope (Zeiss Inc., Thornwood, NY). Images were stored on Sony optical discs and printed on a Tektronix printer (Tektronix, Wilsonville, OR).

Protein Preparations, SDS-PAGE and Western Immunoblotting

Confluent cell cultures were solubilized in sample buffer consisting of 8 M urea, 1% sodium dodecyl sulfate (SDS) in 10 mM Tris-HCl, pH 6.8 and 15% β-mercaptoethanol. DNA was sheared by sonication using a 50 watt Ultrasonic Processor (Vibracell Sonics and Materials Inc., Danbury, CT). Matrix of MCF-10A cells was prepared according to Gospodarowicz (1984) and solubilized in sample buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and processed for immunoblotting according to Zackroff et al. (1984).

Immunoprecipitation

Subconfluent dishes of MCF-10A cells were radiolabeled overnight with 50 μCi/ml of ^{35}S-PRO-MIX cell label (Amersham Corp., Arlington Heights, IL). Conditioned medium of the labeled MCF-10A cells was collected and then pre-cleared by incubation with protein G sepharose beads (Gibco BRL, Gaithersburg, MD) for one hour at 4^{0}C. After centrifugation, monoclonal antibodies were added to the supernatant and the mix was then incubated for 1 hour at 4^{0}C. Protein G sepharose beads were added and the tubes incubated for an additional hour at 4^{0}C. Beads were collected by centrifugation and washed 5 times in TBS (10 mM Tris-HCl, pH 7.4, 145 mM NaCl and 1mM PMSF) containing 1% Triton X-100. Proteins eluted from the beads in sample buffer were processed for SDS-PAGE/autoradiography as well as immunoblotting.

Electron Microscopy

Cells maintained on tissue culture plastic or in matrigel were fixed for a minimum of 30 minutes in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. After being washed three times in 0.1 M sodium cacodylate buffer, cells were post-fixed in 1% OsO_4 containing 0.8% potassium ferricyanide. Preparations were subsequently stained with uranyl acetate, dehydrated in ethanol, and embedded in Epon-Araldite resin (Tousimis Corp., Rockville, MD). Thin sections of embedded material were stained with lead nitrate and sodium citrate and viewed at 60 kV in a JEOL 100CX electron microscope (JEOL USA, Peabody, MA).

RESULTS

MCF-10A Cells Express Laminin-5 as well as Hemidesmosomal Proteins

MCF-10A cells were maintained on glass coverslips for 24 hours and then processed for immunofluorescence microscopy using monoclonal antibodies against laminin-5. The latter stain in a leopard spot pattern along sites of cell-substrate association as determined by confocal laser scan microscopy (Fig. 5A). Laminin-5 antibody reactivity also occurs along areas of the glass coverslip where there are no apparent cells, suggesting that the MCF-10A cells leave behind "trails" of laminin-5 as they migrate over their substrate. Since MCF-10A cells are derived from human mammary glands, we also determined whether laminin-5 is a component of breast epithelial basement membranes. Indeed, basement membranes encircling groups of breast epithelial cells show strong reactivity with laminin-5 antibodies in cryosections of mammary tissue material (Fig. 5C).

In addition to laminin-5, MCF-10A cells, processed for indirect immunofluorescence microscopy, are recognized by antibodies against major components of hemidesmosomes including both bullous pemphigoid antigens (BP180, BP230) as well as the β4 and α6 integrin subunits (Jones et al., 1994; Green and Jones, 1996)(Fig. 6). All of these antibodies generate similar leopard spot staining patterns along the basal aspect of the adherent cells (Fig. 6). This pattern is comparable to that generated by laminin-5 antibodies (Fig. 5A). However, unlike laminin-5, there is an absence of hemidesmosome protein in areas of the glass coverslips devoid of cells (Fig. 6).

Electron microscopic analyses of MCF-10A cells reveals that they assemble hemidesmosome-like structures where they abut their substrates (Fig. 7). These structures possess all of the morphological features of hemidesmosomes observed in mammary epithelial cells in situ i.e. they have triangular shaped, trilayered cytoplasmic plaques (Fig. 7; Jones et al., 1994; Bergstraesser et al., 1995).

To confirm that MCF-10A cells express hemidesmosome components, we have analyzed cell extracts by immunoblotting using antibodies directed against BP180 and BP230, and antisera against β4 integrin and the "light" chain of α6 integrin (Fig. 8A). These antibodies recognize species of 180, 230, 200 and 30kD respectively (Fig. 8A, lanes 1,3,5 and 7). Furthermore, the MCF-10A hemidesmosomal proteins co-migrate with their epidermal equivalents present in extracts of SCC12 cells (Fig. 8A, lanes 2,4,6 and 8).

MCF-10A Cells Produce a Laminin-5 Rich Matrix and Secrete Soluble Laminin-5

We have analyzed both the matrix deposited onto substrates by MCF-10A cells as well as MCF-10A conditioned medium for the presence of laminin-5 using a combination of immunoblotting and immunoprecipitation. MCF-10A matrix was prepared according to the procedure of Gospodarowicz (1984). This matrix contains four prominent polypeptides of 155, 135, 100 and 80kD and is rich in subunits of laminin-5 as shown by immunoblotting using a monoclonal antibody which recognizes the β2 135kD laminin-5 subunit (Fig. 8B). In addition, the 155, 135 and 100kD species present in MCF-10A matrix co-migrate with the major polypeptides

immunoprecipitated from MCF-10A conditioned medium by two laminin-5 monoclonal antibodies (GB3 and C2-9)(Fig. 8C, lanes 1 and 3). The 135kD polypeptides immunopreciptated from MCF-10A conditioned medium by both these anti-laminin-5 monoclonal antibodies are recognized by the β2 chain antibody in immunoblots (Fig. 8C, lanes 2 and 4).

MCF-10A Cells Undergo Branching Morphogenesis when Plated on Matrigel

When MCF-10A cells are embedded into liquid matrigel, which is then allowed to gel, they remain as discrete cellular aggregates ("acini") for 7 days or more regardless of cell concentration (Howlett et al., 1995). In contrast, MCF-10A cells form an interconnected set of tube-like structures, one day after being plated at a concentration of $2.5x10^4$ cells/cm^2 on top of matrigel (Fig. 9A). These are similar to the networks of HMECs observed in matrigel and collagen I gels (Bergstraesser and Weitzman, 1996; Berdichevsky et al., 1994).

The ability of MCF-10A cell to assemble into tube-like arrays is cell concentration dependent. At cell concentrations of $1.25x10^4/cm^2$ or below the MCF-10A cells remain as small aggregates on the matrigel (Fig. 9B). Indeed, they remain in similar aggregates even at 7 days following plating (result not shown).

The tube-like multicellular aggregates of MCF-10A cells in matrigel were processed for confocal immunofluorescence microscopy using antibodies against laminin-5, α6 integrin and α3 integrin (Fig. 10). Both laminin-5 and α6 integrin are concentrated along the edges of the MCF-10A tubes where the cells abut matrigel (Fig. 10A,B). α3 integrin is localized at the latter sites although it is also present at areas of cell-cell contact (Fig. 10C). An IgG control fails to stain the cell population in Fig. 10D.

Antibody Inhibition of MCF-10A Morphogenesis

We next used an immunological approach to assess the potential role of laminin-5 and its receptors (the integrins α6β4 and α3β1) in matrigel induced branching morphogenesis of MCF-10A cells. For these studies MCF-10A cells were incubated for 15 minutes at 37^0C in medium containing either control IgG (50 μg/ml) or in function blocking antibodies directed against α6 integrin (GoH3 at 50 μg/ml), α3 integrin (P1B5 diluted 1:20) and laminin-5 (C2-9 diluted 1:5)(Fig. 11). The cells in the antibody containing medium were plated onto matrigel coated surfaces at $2.5x10^4/cm^2$. After 24 hours the cells incubated in control IgG had formed long interconnected tubes whereas there was an obvious inhibition of branching morphogenesis in cultures which had been incubated in the α3 and α6 integrin antibodies as well as those cells incubated with the laminin-5 antibodies (Fig. 11).

We also fixed and processed the antibody treated cells for electron microscopy. We analzyed at least twenty MCF-10A cells in contact with matrigel under each experimental condition (Fig. 12). MCF-10A cells plated onto matrigel in the presence of control IgG assemble hemidesmosomes at sites of cell-matrigel association (Fig. 12A). The latter appear as electron dense structures with extracellular sub-basal dense plates which indicate formation of "mature"

hemidesmosomes (Fig. 12A, inset). In contrast, no hemidesmosome were observed along regions of cell-matrigel interaction in cultures incubated in function blocking α3 integrin, α6 integrin and laminin-5 antibodies (Fig. 12B-D).

SUMMARY AND DISCUSSION

In this study we have shown that MCF-10A cells, an immortalized mammary epithelial cell line, like HMECs, derived from reduction mammoplasties, undergo a branching morphogenesis when maintained on top of matrigel (Bergstraesser and Weitzman, 1996). This phenomenon is highly dependent on cell concentration. We have never observed the formation of tubular arrays when MCF-10A cells are plated onto matrigel at concentrations below $1.25x10^4$cells/cm^2. Just a two fold increase in this cell number is enough to trigger a matrigel induced branching morphogenesis of the MCF-10A cells. Indeed, we find it remarkable that within 1 day of plating onto matrigel, MCF-10A cells assemble into an anastomosing network, organized into a branching pattern much like that seen in vivo in postpubertal mammary glands (Daniel and Silberstein, 1987). This type of pattern has been observed by Berdichevsky et al. (1994) when the human mammary cell line HB-2 is maintained in collagen type I gels.

HMECs assemble hemidesmosomes in vivo (Watson et al., 1988). In vitro they are also capable of forming hemidesmosomes, although this generally takes up to 14 days following plating on tissue culture substrates (Bergstraesser et al., 1995). Like HMECs in vivo, MCF-10A cells express the major components of hemidesmosomes as determined by immunofluorescence, immunoblotting and immunoprecipitation. Moreover, MCF-10A cells readily assemble hemidesmosome-like structures within 24 hr after plating onto uncoated glass coverslips i.e. much faster than their normal counterparts. The speed of hemidesmosome appearance in MCF-10A cells was the more surprising since earlier work had suggested that MCF-10A cells were unable to assemble bona fide hemidesmosomes in vitro (Tait et al., 1990).

When maintained on matrigel, MCF-10A cells assemble hemidesmosomes at sites of cell-matrigel interaction. Consistent with this, a hemidesmosome associated matrix component and its receptor, namely laminin-5 and $\alpha6\beta4$ integrin, are distributed at sites of MCF-10A cell-matrigel interaction. Such observations triggered our interest in the potential role of hemidesmosome components in branching morphogenesis of MCF-10A cells. Since it is already established that laminin-5 and $\alpha6\beta4$ integrin heterodimer are essential for hemidesmosome assembly, we have been able to assay the role of hemidesmosomes in branching morphogenesis of MCF-10A cells by using antibodies which inhibit both the activities of laminin-5 and $\alpha6\beta4$ integrin (Jones et al., 1991; Kurpakus et al., 1991; Spinardi et al., 1995; van der Neut et al., 1996; Georges-Labouesse et al., 1996; Baker et al., 1996b).

Function blocking antibodies directed against laminin-5 not only prevent hemidesmosome assembly in MCF-10A cells maintained on matrigel but also significantly inhibit branching morphogenesis. Similarly, antibody GoH3, which blocks $\alpha6$ integrin function, inhibits both hemidesmosome formation and MCF-10A morphogenesis. Since the $\alpha6$ integrin subunit is known to preferentially bind $\beta4$ integrin in cells which coexpress both of its $\beta1$ and $\beta4$ integrin partners, as is the case in MCF-10A cells, the inhibitory effects of GoH3 antibodies on MCF-10A cells

likely impact the function of the hemidesmosome-associated α6β4 integrin heterodimer (Giancotti et al., 1992; Baker and Jones, unpublished observations).

Indeed, we assume that matrigel, or more specifically its laminin-1 component, provides an initial framework for MCF-10A attachment and triggers a series of morphogenetic events (Streuli et al., 1995). This includes secretion of laminin-5 which then induces the MCF-10A cells to nucleate the assembly of their own hemidesmosomes, a process requiring laminin-5/α6β4 integrin interaction. We suggest that the formation of the latter complex is necessary to complete branching morphogenesis.

The idea that hemidesmosomes may be involved in morphogenetic events is supported indirectly by recent reports which indicate that hemidesmosomes are sites of signal transduction (Maniero et al., 1995; Maniero et al., 1996). For example, the β4 subunit of the α6β4 hemidesmosome associated integrin possesses an unusually long cytoplasmic tail which is associated with one or more protein kinases (Tamura et al., 1990; Maniero et al., 1995). The latter are believed to be involved in a matrix induced cascade of phosphorylation events resulting in phosphorylation not only of the β4 integrin subunit but also of a recently identified protein of 80kD (Xia et al., 1996; Maniero et al., 1995).

Laminin-5 and α6 antibodies are not exclusive in their abilities to block morphogenesis of MCF-10A cells in matrigel. A function perturbing α3 integrin antibody, P1B5, is also capable of inhibiting matrigel induced branching morphogenesis of MCF-10A cells. The α3β1 integrin heterodimer is not a component of the hemidesmosome but, like α6β4 integrin is a receptor for laminin-5 (Carter et al., 1990; Carter et al., 1991). In in vitro assays, it has been shown that cell interaction with laminin-5 is initiated by the α3β1 integrin heterodimer (Carter et al., 1991). Subsequently laminin-5 appears to "switch" receptors and binds to the α6β4 integrin as a prelude to hemidesmosome assembly (Carter et al., 1990; Carter et al., 1991; Spinardi et al., 1995; Xia et al., 1996). Thus one explanation for the morphogenetic impact of the α3 integrin blocking antibody is that P1B5 inhibits the interaction of cells with their own laminin-5. However, we cannot discount that α3 integrin is involved in cell binding to the laminin-1 component of matrigel (Streuli et al., 1995). Of course, P1B5 may inhibit both laminin-1 and laminin-5 interactions of the MCF-10A cells.

In summary, we have identified a model system and a continuous cell line, MCF-10A, for the study of the role of hemidesmosome matrix and integrin components in tissue morphogenesis. In this model, matrigel provides a three dimensional environment which triggers a series of cellular morphogenetic events, involving the assembly of hemidesmosomes and expression of hemidesmosome matrix and integrin components, in MCF-10A cells. Indeed, it is becoming clear that the hemidesmosome is not simply a spot weld to tether cells to connective tissue but, through the functional properties of its components, the hemidesmosome can have profound impact on the differentiation and organization of epithelia at the tissue level.

Figure Legends

Figure 1. FG cells were plated into control medium and 24 hr later processed for immunofluorescence using monoclonal antibody GoH3 to the α6 integrin subunit (A). Confocal microscopy shows that α6 staining consists of patches and streaks in a plane of focus located at the basal aspect of the cell. B, phase contrast image. Bar, 25 μm.

Figure 2. A. Approximately 10 μg of extracts of FG cells maintained for 24 hr in their normal medium (lane 1) or for 6 hr in laminin-5 rich medium (lane 2) were prepared for SDS-PAGE on 12% gels. Separated polypeptides were then transferred to nitrocellulose and subsequently processed for immunoblotting using 4E9G8 antibody. The latter shows reactivity with a 25 kD protein only in lane 2. Molecular weight standards indicated by dashes are 200, 97, 66, 45, 31 and 21 kD.
B. The α6A integrin cytoplasmic non-phospho- and phospho-peptides at 1 mg/ml or 0.1mg/ml (from left to right) were spotted onto PVDF membrane. The PVDF pieces were then processed for immunoblotting either using 4E9G8 monoclonal antibody or serum 6844. Both peptides are recognized by antibodies in the 6844 serum whereas 4E9G8 fails to recognize the phospho-peptide.
C. FG cells, maintained in the absence (-LN5) or presence (+LN5) of rat laminin-5, were labeled with ^{32}P. The α6 integrin subunit was then immunprecipitated from the labeled cells using monoclonal antibody GoH3. Equal amounts of precipitated α6 integrin were subjected to SDS-PAGE and were either transferred to nitrocellulose and processed for immunoblotting using the α6A integrin serum, 6844, or visualized by autoradiography. The reactivity of the 6844 antibodies on the immunoblot indicates that equal amounts of α6A integrin from the FG cells have been loaded onto the two lanes. This was confirmed by scanning densitometry. In contrast, the autoradiograph shows an apparent 37% decrease in the level of phosphorylation of α6A in the GoH3 antibody precipitates derived from FG cells maintained in the presence of laminin-5 (arrow). The asterisk marks cross reactivity of the blotting secondary antibody with the precipitated IgG. Molecular weight standards are (from top to bottom) 97, 66, and 45kD

Figure 3. At 24 hr following plating, FG cells maintained in their normal medium were processed for indirect immunofluorescence using the α6 monoclonal antibody 4E9G8 (A). The antibody generates no obvious staining pattern (the focal plane shown is close to the substratum attached surface of the cells). In contrast, in FG cells maintained for 6 hr in laminin-5 rich medium, antibody 4E9G8 generates staining in circles (C). These circles co-localize with circles stained by GoH3 antibodies (open arrow, E and F). However, it should be noted that GoH3 staining is more extensive than that of 4E9G8 antibodies (closed arrows, E and F). B, D and G show phase contrast images of the cells. Bar in B, 10 μm; bar in G, 25 μm.

Figure 4. Cryosections of normal (A-F) and invasive tumor-containing (G-L) breast tissues were processed for double label immunofluorescence microscopy using GoH3

antibodies (A,D,G and J) in combination with either 4E9G8 (B and H) or GB3 (E and K) antibodies. Sections shown in A-C and D-F, as well as the sections shown in G-I and J-L, were collected consecutively from the microtome. This allowed us to photograph the same islands of normal (and tumor cells) in the distinct double labels. Note that in A,B and D,E all of the antibodies generate similar staining, concentrated at regions of epithelial cell-matrix interaction. In G and J, GoH3 antibodies recognize an island of tumor cells in the absence of corresponding 4E9G8 (H) and GB3 (K) staining. C,F,I and L, phase images; bar, 25 μm.

Figure 5. Laminin-5 is expressed by MCF-10A cells and in human breast tissue. MCF-10A cells were cultured on glass coverslips and processed for indirect immunofluorescence microscopy using the laminin-5 monoclonal antibody (GB3)(A). The cells were viewed by confocal microscopy, the plane of focus being close to the cell-substrate interface. The laminin-5 antibodies stain in a typical leopard spot pattern. The GB3 antibodies also stain areas where there are no apparent cells (arrow). (C) A cryosection of human breast tissue from a reduction mammoplasty was processed for immunofluorescence with GB3 antibodies. These stain the basement membrane zones of islands of epithelial cells. Panels B and D show phase contrast images. Bars, 10μm.

Figure 6. Hemidesmosomal proteins are expressed by MCF-10A cells as shown by indirect immunofluorescence. MCF-10A cells, maintained on glass coverslips were processed for indirect immunofluorescence microscopy using antibodies specific for BP180 (J17)(A), BP230 (10C5)(C), β4 integrin (3E1)(E), and α6 integrin (GoH3)(G). In all cases the antibodies generate a patchy, leopard spot stain along the region of cell-coverslip interaction. B,D,F and H show phase contrast images of the cells. Bar, 10μm.

Figure 7. MCF-10A cells assemble hemidesmosomes when maintained in vitro. This electron micrograph shows a cross section of MCF-10A cells. Arrows indicate numerous electron dense hemidesmosome structures. These possess tripartite cytoplasmic plaques (inset, arrow). Bar, 500nm. Bar in inset, 250nm.

Figure 8. A. Hemidesmosomal proteins are expressed by MCF-10A cells as shown by immunoblotting. MCF-10A cell extracts (lanes 1,3,5 and 7) and extracts of SCC12 cells, a keratinocyte line, (lanes 2,4,6 and 8) were separated by SDS-PAGE on either 6% (lanes 1-6) or 15% (lanes 7,8) polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with antibodies against BP180 (J17, lanes 1,2), BP230 (10C5, lanes 3,4), β4 integrin (6945, lanes 5,6), or the "light" chain of α6 integrin (6845, lanes 7,8).
B. MCF-10A cells deposit laminin-5 on their substrate. MCF-10A matrix was collected according to Gospodarowicz (1984), processed for SDS-PAGE on a 6% gel, and either silver stained (lane 1) or transferred to nitrocellulose and immunoblotted with a monoclonal antibody (clone 17) against the β chain of laminin-

5 (lane 2). In the silver stained preparation, there are prominent polypeptides at 150, 135 and 100 kD representing the α, β and the γ chains of laminin-5 (lane 1). The 135kD protein in this preparation is recognized by the clone 17 antibody (lane 2).
C. MCF-10A secrete laminin-5 into their medium. The medium conditioned by radio-labeled MCF-10A cells was processed for immunoprecipitation using two monoclonal laminin-5 antibodies (GB3, lanes 1,2; C2-9, lanes 3,4). The immunoprecipitated proteins were analyzed by SDS-PAGE/autoradiography (lanes 1 and 3) or prepared for immunoblotting using clone 17 monoclonal antibody against the β chain of laminin-5 (lanes 2 and 4). The laminin-5 antibodies precipitate three major polypeptides of 150, 135 and 100kD (lanes 1,3). The 135kD protein is recognized by the clone 17 antibody (lanes 2,4). Note that there is some breakdown of the laminin-5 in the C2-9 antibody precipitate (lane 2). This may explain the ladder of proteins recognized by the clone 17 antibody in lane 4. The low molecular weight reactive species in lanes 2 and 4 are due to cross reactivity of the secondary antibody anti-mouse IgG with the immunprecipitated mouse IgG.
Dashed lines on the left side of panels A, B, and C indicate weight standards of 200, 116. 97.4, and 66 kD. Dashed lines on the right side of A indicate standards of 66, 45, 31, 21.5 and 14.5 kD. Each lane of the gels was loaded with approximately 10 μg of protein.

Figure 9. MCF-10A cells undergo branching morphogenesis on matrigel in a cell concentration dependent manner. $2.5x10^4/cm^2$ (A) and $1.25x10^4/cm^2$ (B) MCF-10A cells were plated onto matrigel which had been used to coat 35mm dishes. At 24 hours following plating, the cells in A have undergone a branching morphogenesis while the cells in B appear in small aggregates. Bar, 500μm.

Figure 10. Laminin-5 and its receptors are expressed by MCF-10A cells undergoing morphogenesis on matrigel. MCF-10A cells maintained in matrigel for 24 hours were processed for indirect confocal immunofluorescence with monoclonal antibodies recognizing laminin-5 (GB3, A), α6 integrin (GoH3, B), α3 integrin (P1B5, C), or an IgG control (D). Note that the antibodies in A,B and C show staining along regions of cell-matrigel interaction. The insert in (C) is a higher magnification of the boxed area and reveals that α3 integrin occurs at sites of cell-cell as well as cell-matrigel interaction. Bar in A, 100μm; Bar in C, 25μm.

Figure 11. Branching morphogenesis of MCF-10A cells on matrigel is inhibited by antibodies against laminin-5, α6 integrin and α3 integrin. MCF-10A cells were plated onto matrigel in the presence of control IgG (A), or antibodies which block the function of α6 integrin (GoH3, B), α3 integrin (P1B5, C), or laminin-5 (C2-9, D). After 24 hours, the cells in A appear organized into a highly branched array, while those incubated with blocking antibodies remain either as single cells or in small multicellular clusters (B,C and D). The large dark circles in each of the micrographs is an optical artifact. Bar, 100μm.

Figure 12. MCF-10A cells assemble hemidesmosomes on matrigel but this is inhibited by integrin and laminin-5 antibodies. MCF-10A cells were plated onto matrigel in the presence of control IgG (A), or antibodies which block the function of α6 integrin (GoH3, B), α3 integrin (P1B5, C), or laminin-5 (C2-9, D). After 24 hours the cells on matrigel were fixed for electron microscopy. Note that in A there are three hemidesmosome-like structures along the region of cell-matrigel interaction (arrows). One of these (in the box) is shown at higher power in the inset. It possesses an electron dense cytoplasmic plaque and also a sub-basal dense plate. There are no obvious hemidesmosomes in cells in contact with matrigel in B-D (higher power views of these regions are shown in the insets). m, matrigel. Bar in A, 500nm; Bar in inset 60nm.

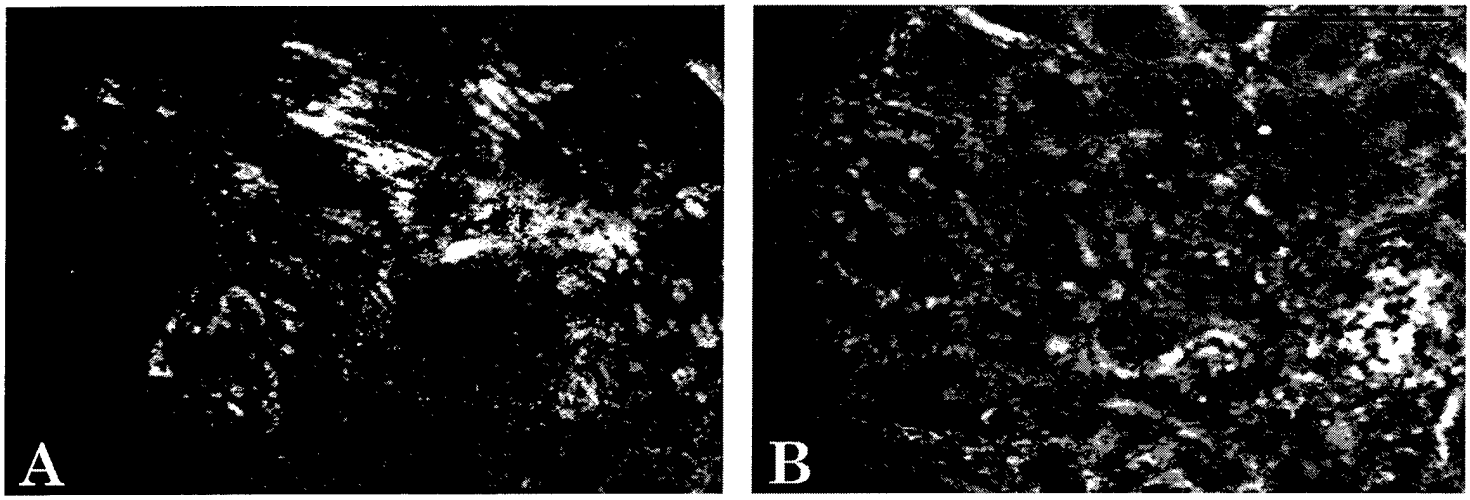
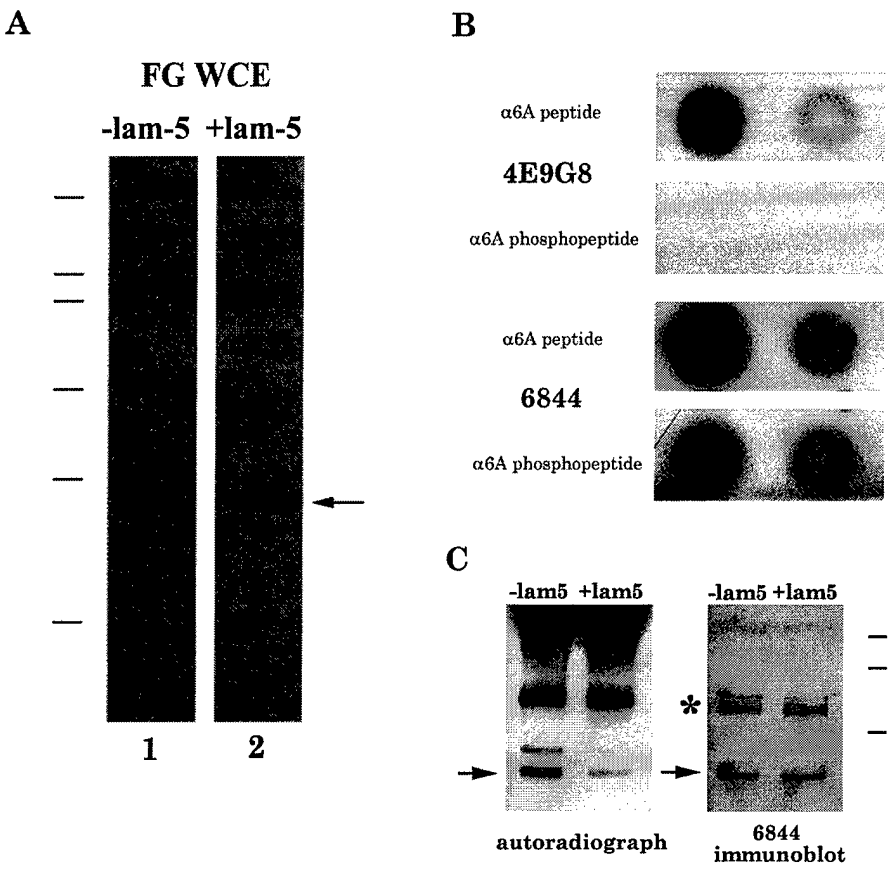


Figure 1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1
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B
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autoradiograph
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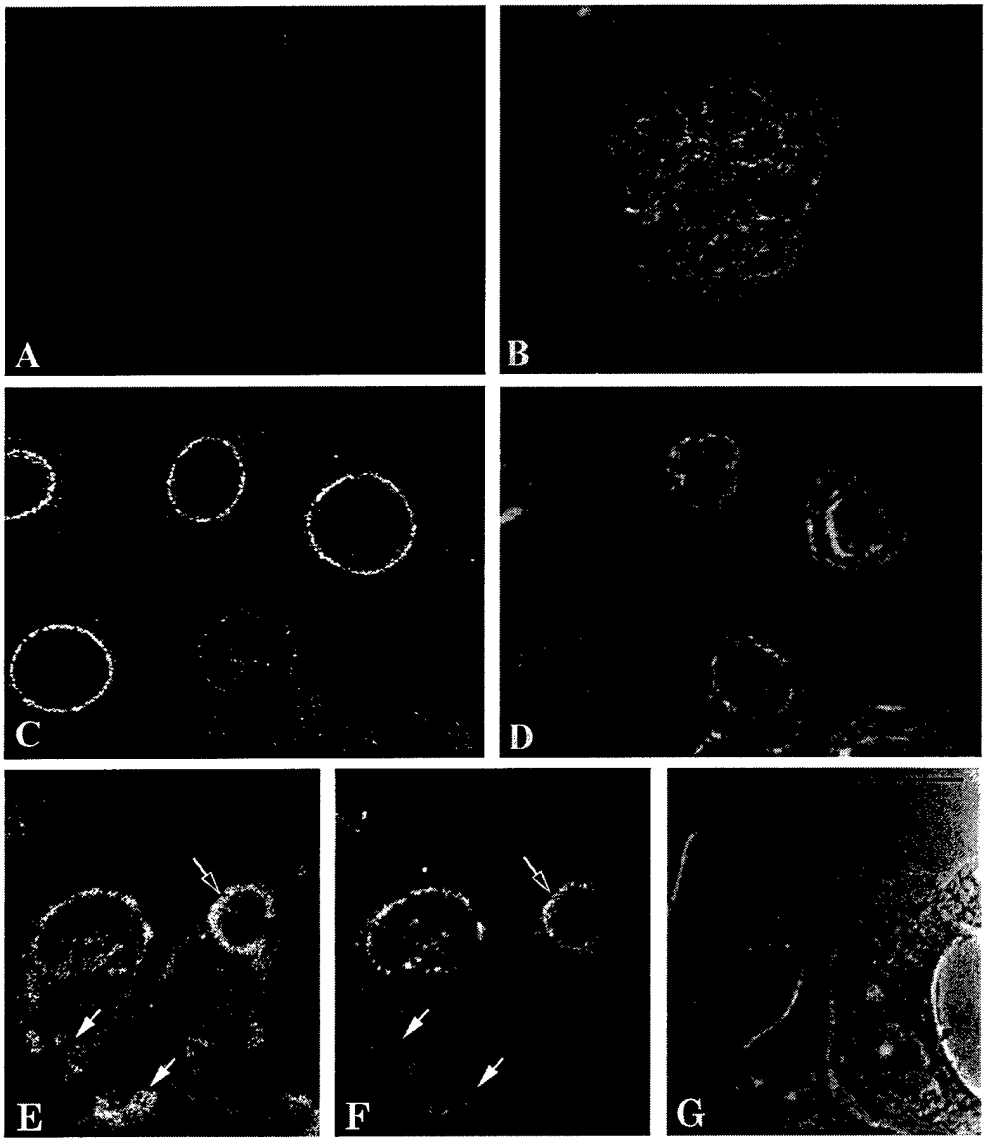
immunoblot

Figure 2



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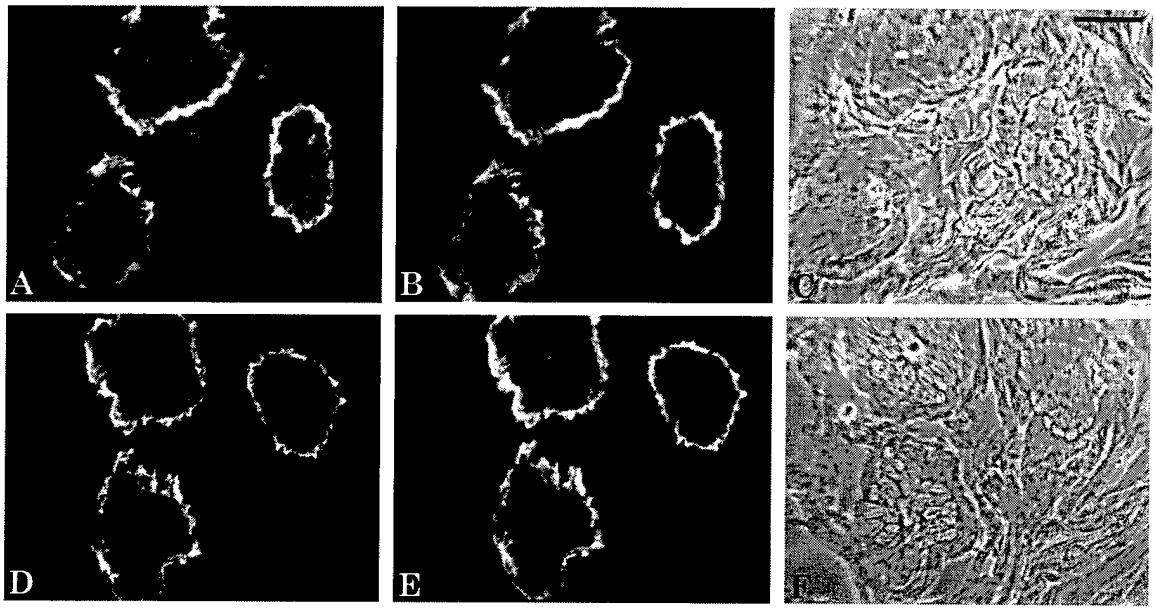


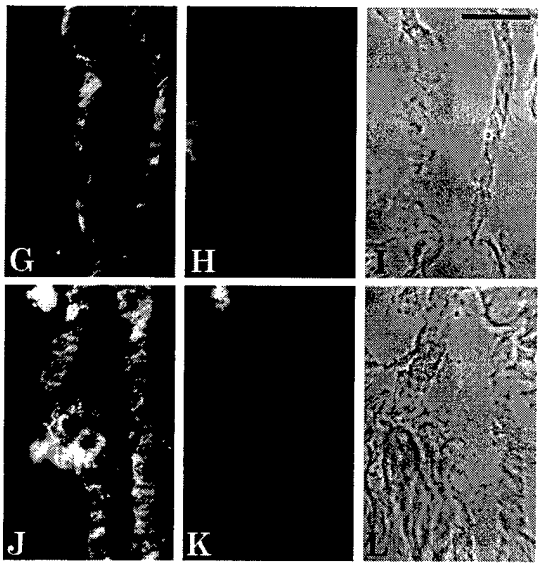
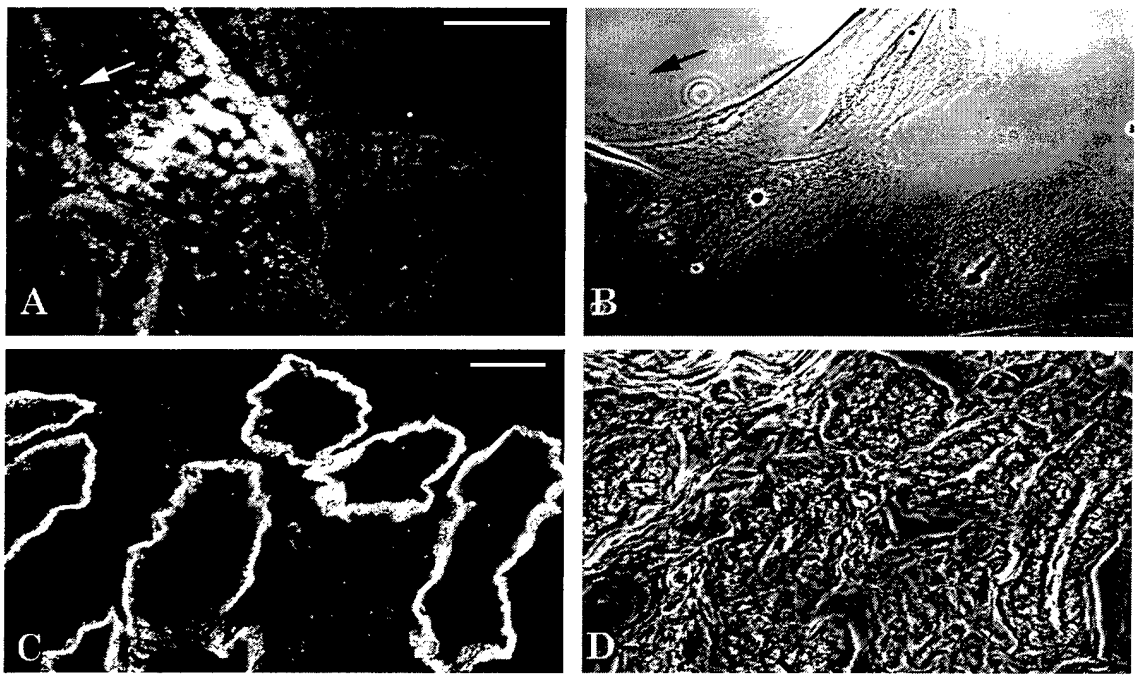


Figure 4



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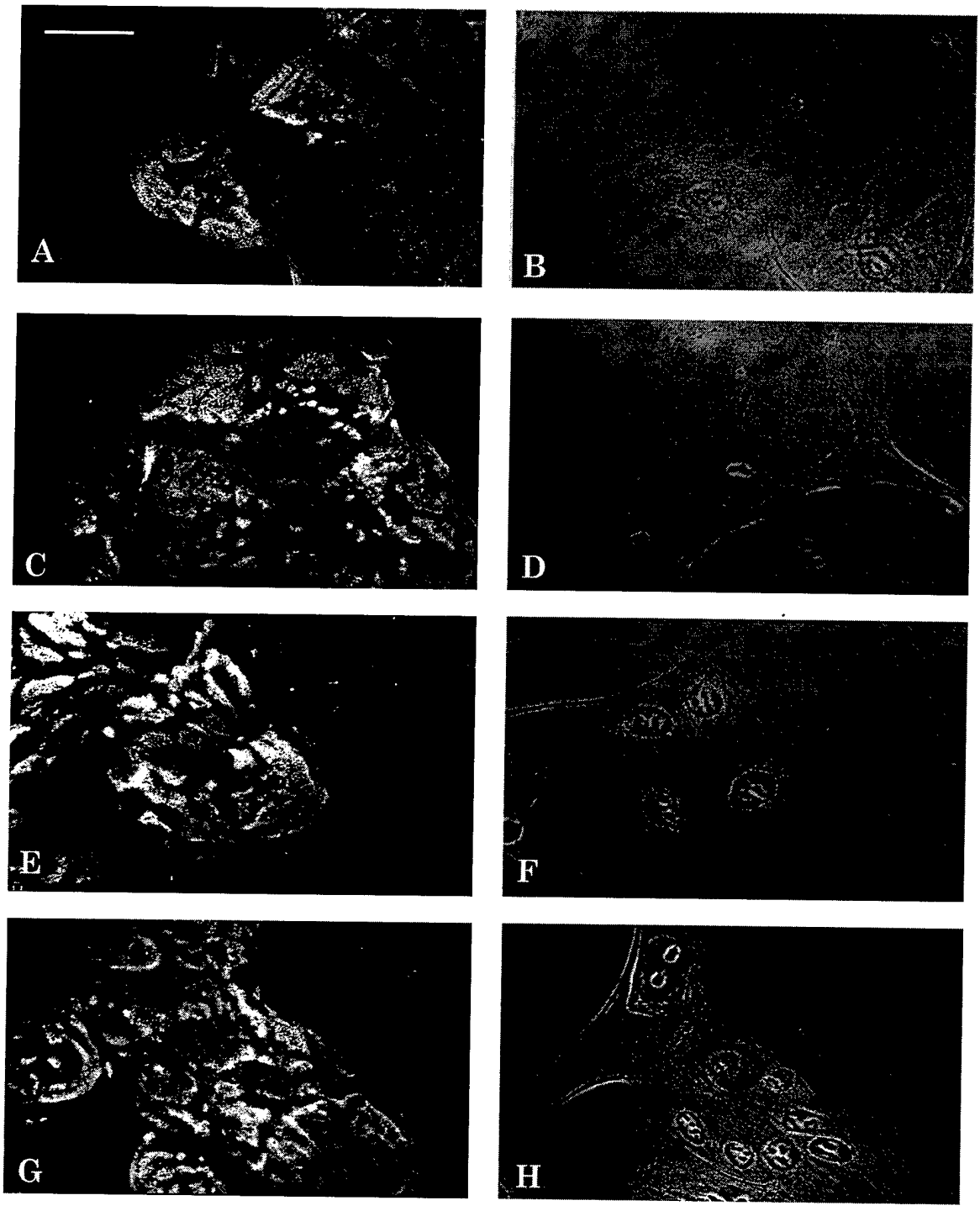


Figure 6

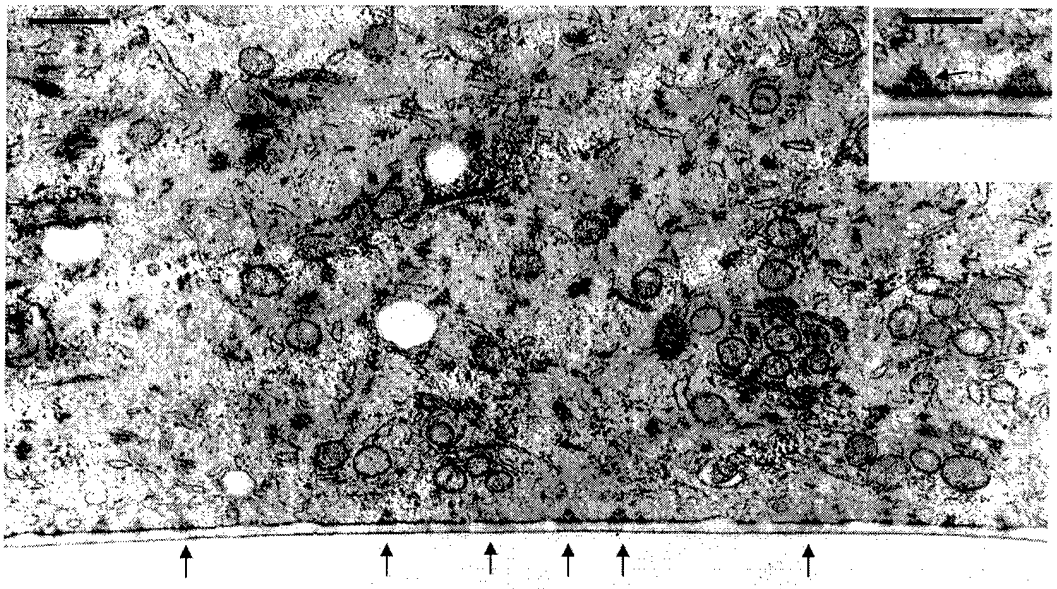


Figure 7

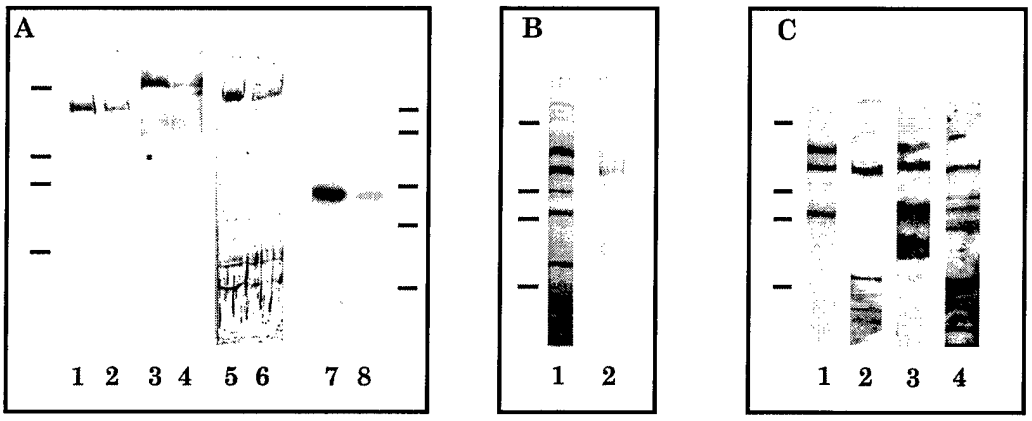


Figure 8

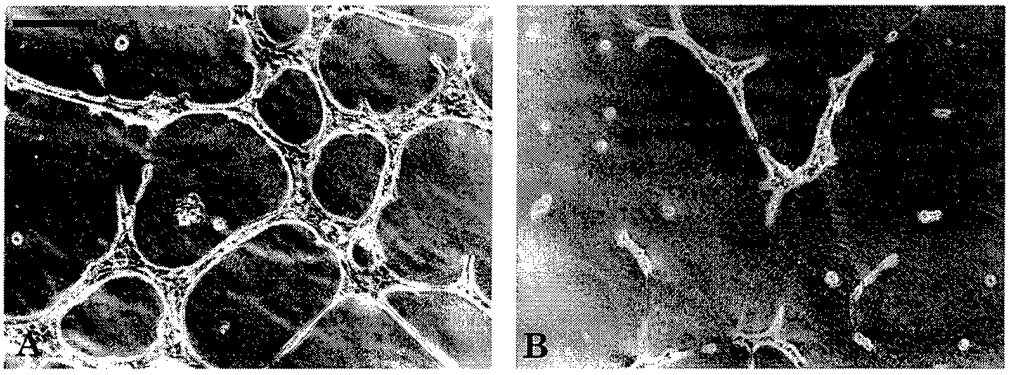


Figure 9

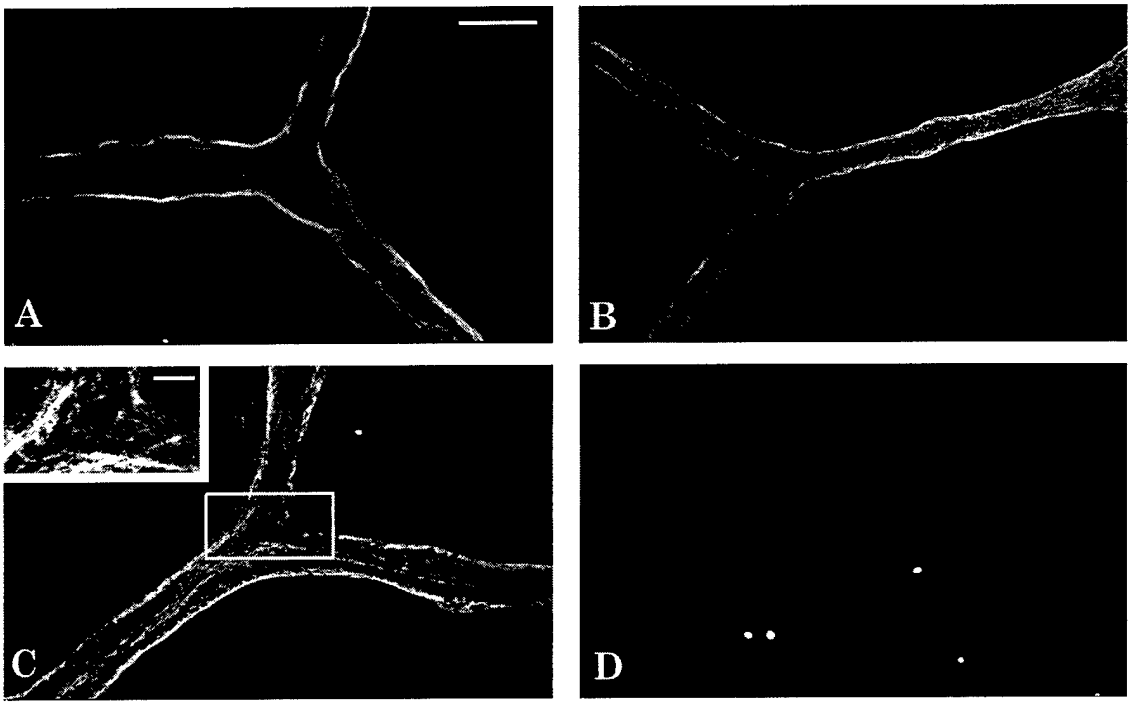
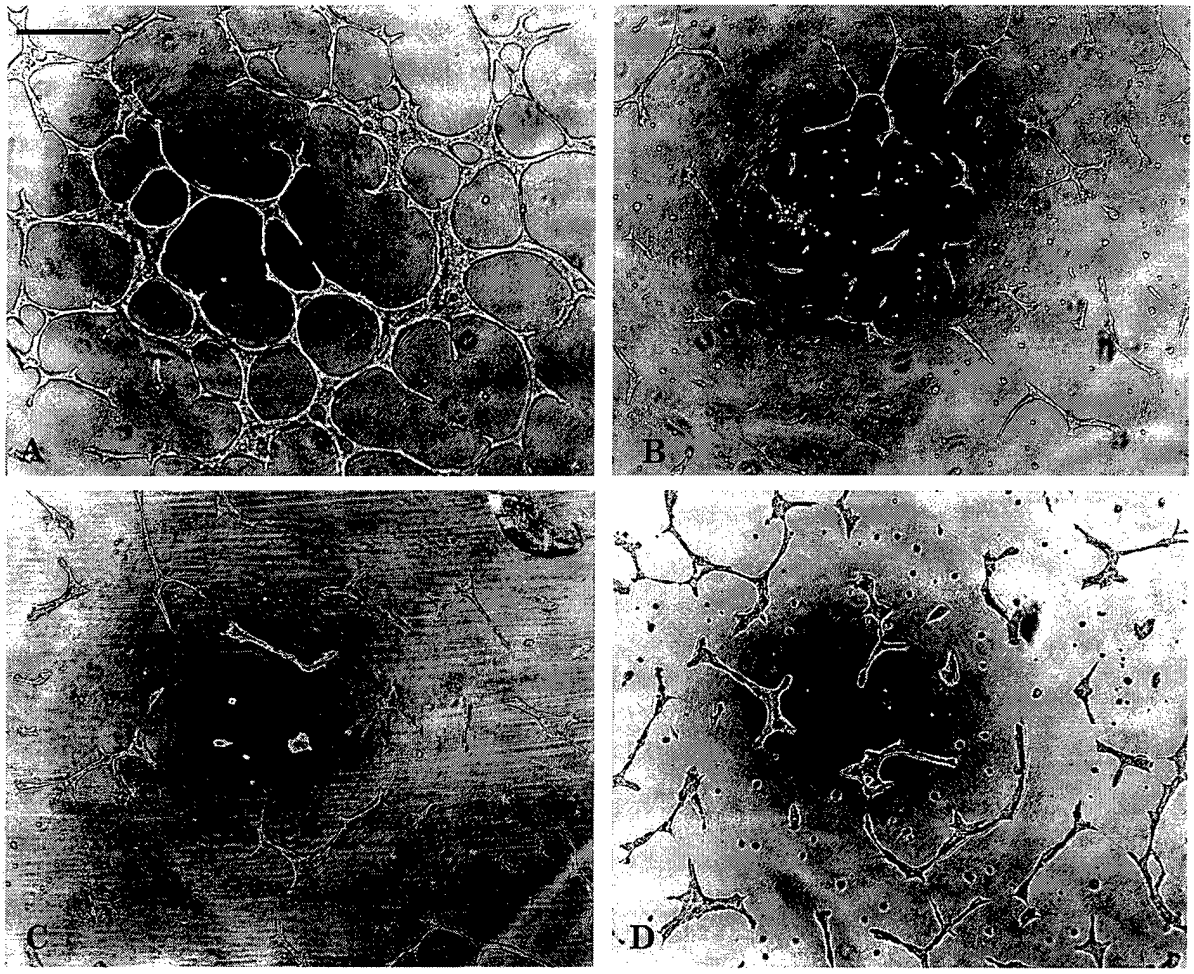


Figure 10



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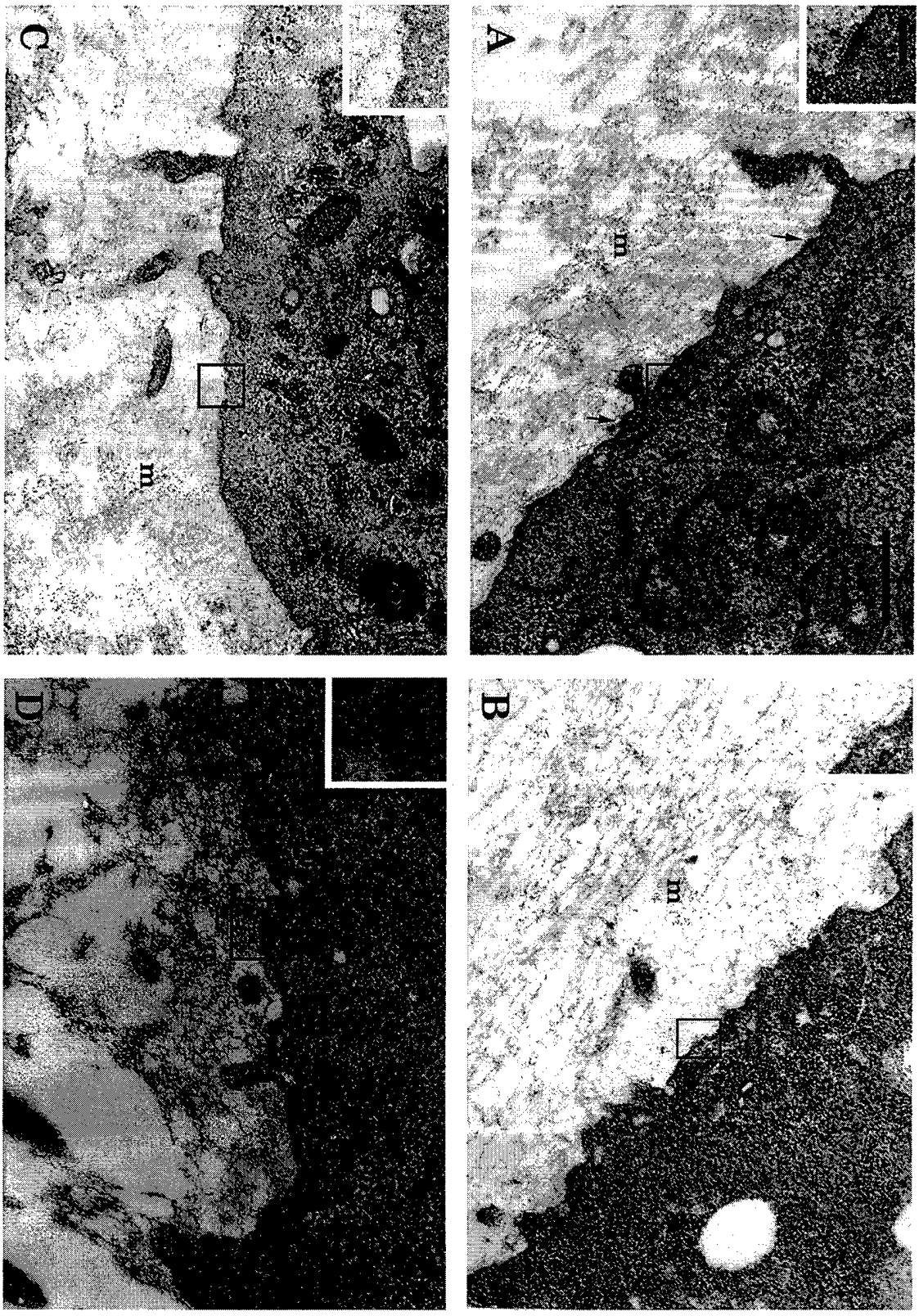

Figure 12

Future Studies for the Next Grant Period

1. We will continue to analyze tumor tissue with antibodies against phosphorylated and non-phosphorylated epitopes of α6 integrin to determine their potential in differentiating invasive and non-invasive cancers.
2. We will begin to undertake Task 2 in which hemidesmosome message levels will be analyzed in normal and tumor tissue.
3. We will investigate the fate of primary normal and tumor breast epithelial cells in the same model system that we detail in Stahl et al. (in press) i.e. cells maintained on matrigel. In the case of tumor cells we will also assess whether the addition of exogenous laminin-5 "rescues" their ability to assemble three dimensional stuctures such as those described in Stahl et al. (in press)(Fig. 9).

(7) **Conclusions**

1. Certain hemidesmosome antibody probes (those that can differentiate between phosphorylated and non- phosphorylated α6 integrin) may be useful in differentiating non-invasive from invasive carcinoma cells.
2. MCF-10A cells assemble hemidesmosomes in vitro and undergo branching morphogenesis in matrigel. Indeed, they provide an ideal model for morphogenesis studies.
3. Branching morphogenesis of MCF-10A cells is inhibited by antibodies to laminin-5 and its receptors indicating a role for hemidesmosomes in normal breast tissue development.

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(9) **Appendix**

We include a copy of the paper entitled "Expression of hemidesmosomes and component proteins is lost by invasive breast cancer cells" (Bergstraesser et al., 1995) published in the American Journal of Pathology, a copy of a review article on hemidesmosomes published in the FASEB journal (Green and Jones, 1996) and a preprint of a manuscript accepted for publication by the Journal of Cell Science (Stahl et al.) as part of our progress report.

page nos: MS order: Page total: Reprints: Start page:
Journal of Cell Science 1, 000-000 (199)
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The role of laminin-5 and its receptors in mammary epithelial cell branching morphogenesis

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SUMMARY

In vivo, normal mammary epithelial cells utilize hemidesmosome attachment devices to adhere to stroma. However, analyses of a potential role for hemidesmosomes and their components in mammary epithelial tissue morphogenesis have never been attempted. MCF-10A cells are a spontaneously immortalized line derived from mammary epithelium and possess a number of characteristics of normal mammary epithelial cells including expression of hemidesmosomal associated proteins such as the two bullous pemphigoid antigens, α6β4 integrin and its ligand laminin-5. More importantly, MCF-10A cells readily assemble mature hemidesmosomes when plated onto uncoated substrates. When maintained on matrigel, like their normal breast epithelial cell counterparts, MCF-10A cells undergo a branching morphogenesis and asseml hemidesmosomes at sites of cell-matrigel interactio Function blocking antibodies specific for human laminin and the α subunits of its two known receptors (α3β1 ai α6β4 integrin) not only inhibit hemidesmosome assemb by MCF-10A cells but also impede branching morphoge esis induced by matrigel. Our results imply that tl hemidesmosome, in particular those subunits comprisi its laminin-5/integrin 'backbone', play an important role morphogenetic events. We discuss these results in light recent evidence that hemidesmosomes are sites involved signal transduction.

Key words: Hemidesmosome, Integrin, Laminin

INTRODUCTION

Extracellular matrix plays a crucial role in determining the morphogenesis of a number of epithelial tissue types (Hay, 1993). One of the most dramatic examples of this phenomenon is the regulation of mammary epithelium phenotype by elements of basement membranes derived from the Engelbreth-Holm-Swarm tumor (matrigel) (Bissell and Ram, 1989; Barcellos-Hoff et al., 1989; Blum et al., 1989; Lin and Bissell, 1993). Indeed, mouse mammary epithelial cells assemble into structures remarkably similar to alveoli of lactating mammary glands and produce milk proteins when maintained in matrigel (reviewed by Lin and Bissell, 1993).

Compared with the rodent system, analyses of morphogenesis of human mammary epithelial cells has progressed more slowly, in part because of difficulties in maintaining cultures of primary human cells. This problem has been partially alleviated by the development of media for the culture of primary human mammary epithelial (HMEC) cells although establishment of primary cultures remains problematic (Stampfer, 1985; Bergstraesser and Weitzman, 1993). One alternative is the use of continuous human mammary epithelial cell lines such as MCF-10A (Soule et al., 1990). Indeed, a model for the study of mammary epithelial cell morphogenesis using MCF-10A cells has recently been described (Howlett et al., 1995).

It has now been shown that laminin-1 is the matrix component of matrigel which regulates morphogenesis as well as milk protein expression of mouse mammary epithelial cells in vitro (Streuli et al., 1995). Furthermore, the domain respo sible for such regulation resides in the so-called E3 fragme of laminin-1 and is located towards the carboxy terminus the α1 subunit of the heterotrimer (Streuli et al., 1995 Laminin-1, via its cell surface receptors, is believed to establis polarity of mammary epithelial cells, a process which is a essential prerequisite to cell differentiation (Streuli et al 1995). However, following polarization, it is hypothesized th epithelial cells modulate their own microenvironment b producing additional basement membrane components (Bisse and Ram, 1989). The latter could include a number of laminin since laminin-1 is only one of several laminin isoforms whic occur in intact basement membranes (Timpl and Brown, 1994 For example, laminin-5 is widely distributed in the basemen membranes of epithelial tissues, including the mammary glanc as we show here (Verrando et al., 1987; Rousselle et al., 1991 Carter et al., 1991; Kallunki et al., 1992; Timpl and Brown 1994). Do these endogenously secreted basement membran elements play a role in mammary epithelial morphogenesis To answer this question, we have analyzed the function o laminin-5 in an in vitro model of mammary epithelial mor phogenesis using MCF-10A cells. These cells underg branching morphogenesis (i.e. assemble a highly anastomosec multicellular network), when cultured on matrigel. We show that matrigel-induced differentiation of MCF-10A cells i inhibited by function blocking laminin-5 antibodies as well a antibodies against two distinct laminin-5 receptors. Since laminin-5 is a component of certain cell-matrix junctions called

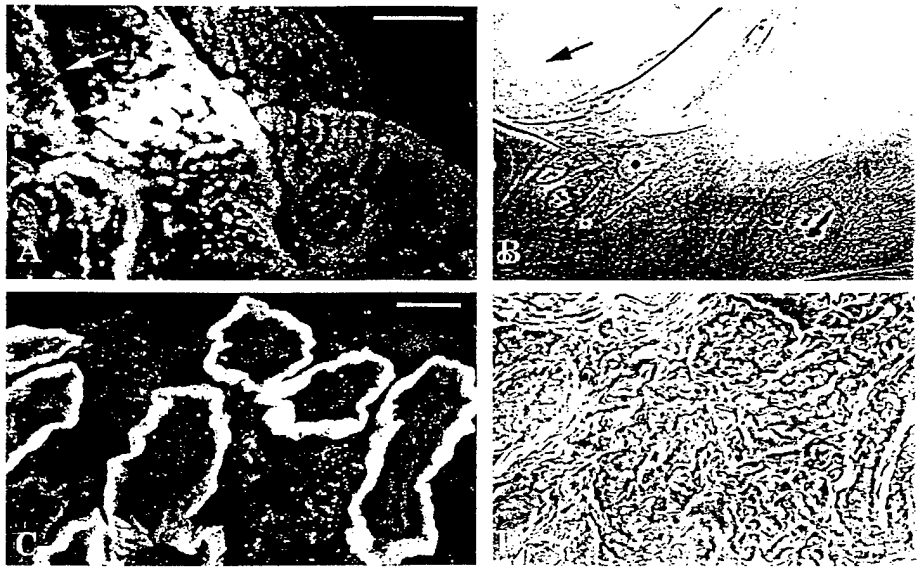
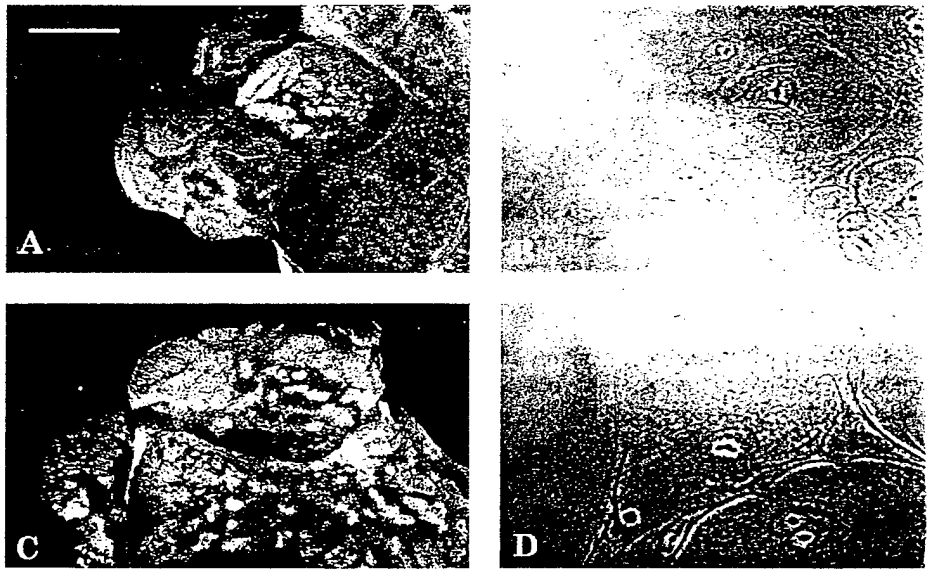


Fig. 1. Laminin-5 is expressed by MCF-10A cells and in human breast tissue. MCF-10A cells were cultured on glass coverslips and processed for indirect immunofluorescence microscopy using the laminin-5 monoclonal antibody (GB3)(A). The cells were viewed by confocal microscopy, the plane of focus being close to the cell-substrate interface. The laminin-5 antibodies stain in a typical leopard spot pattern. The GB3 antibodies also stain areas where there are no apparent cells (arrow). (C) A cryosection of human breast tissue from a reduction mammoplasty was processed for immunofluorescence with GB3 antibodies. These stain the basement membrane zones of islands of epithelial cells. (B and D) Phase contrast images. Bars, 10 μm.



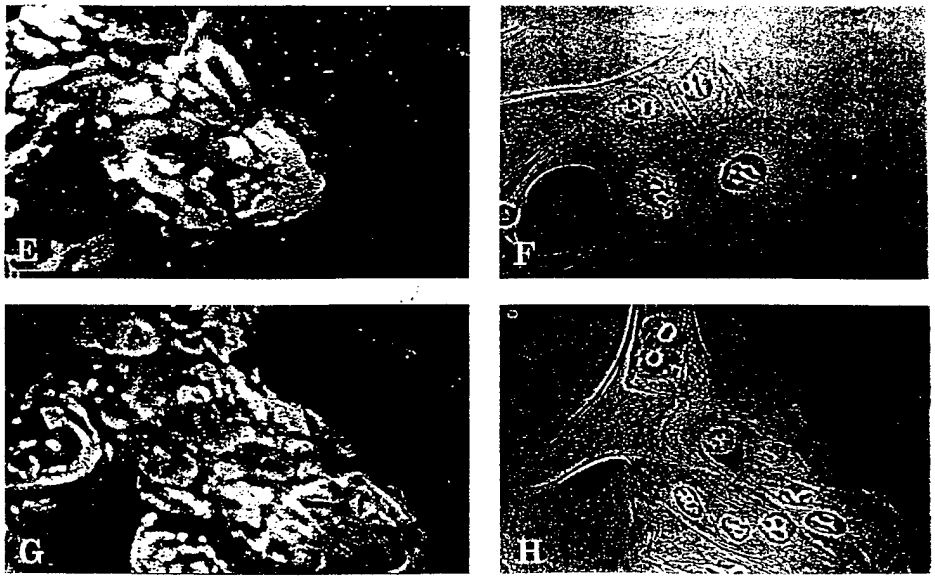


Fig. 2. Hemidesmosomal proteins are expressed by MCF-10A cells as shown by indirect immunofluorescence. MCF-10A cells, maintained on glass coverslips were processed for indirect immunofluorescence microscopy using antibodies specific for BP180 (J17)(A), BP230 (10C5)(C), β4 integrin (3E1)(E), and α6 integrin (GoH3)(G). In all cases the antibodies generate a patchy, leopard spot stain along the region of cell-coverslip interaction. (B,D,F,H) Phase contrast images of the cells. Bar, 10 μm.

breast epithelial cells show strong reactivity with laminin-5 antibodies in cryosections of mammary tissue material (Fig. 1C).

In addition to laminin-5, MCF-10A cells, processed for indirect immunofluorescence microscopy, are recognized by antibodies against major components of hemidesmosomes including both bullous pemphigoid antigens (BP180, BP230) as well as the β4 and α6 integrin subunits (Jones et al., 1994; Green and Jones, 1996) (Fig. 2). All of these antibodies generate similar leopard spot staining patterns along the basal aspect of the adherent cells (Fig. 2). This pattern is comparable to that generated by laminin-5 antibodies (Fig. 1A). However, unlike laminin-5, there is an absence of hemidesmosome protein in areas of the glass coverslips devoid of cells (Fig. 2).

Electron microscopic analyses of MCF-10A cells reveals that they assemble hemidesmosome-like structures where they abut their substrates (Fig. 3). These structures possess all of the morphological features of hemidesmosomes observed in mammary epithelial cells in situ (i.e. they have triangular shaped, trilayered cytoplasmic plaques (Fig. 3; Jones et al., 1994; Bergstraesser et al., 1995).

To confirm that MCF-10A cells express hemidesmosome components, we have analyzed cell extracts by immunoblotting using antibodies directed against BP180 and BP230, and antisera against β4 integrin and the 'light' chain of α6 integrin (Fig. 4A). These antibodies recognize species of 180, 230, 200 and 30 kDa, respectively (Fig. 4A, lanes 1,3,5 and 7). Furthermore, the MCF-10A hemidesmosomal proteins co-migrate with their epidermal equivalents present in extracts of SCC12 cells (Fig. 4A, lanes 2,4,6 and 8).

MCF-10A cells produce a laminin-5 rich matrix and secrete soluble laminin-5

We have analyzed both the matrix deposited onto substrates by MCF-10A cells as well as MCF-10A conditioned medium for the presence of laminin-5 using a combination of immunoblotting and immunoprecipitation. MCF-10A matrix was prepared according to the procedure of Gospodarowicz (1984). This matrix contains four prominent polypeptides of 155, 135, 100 and 80 kDa and is rich in subunits of laminin-5 as shown by immunoblotting using a monoclonal antibody which recognizes the β2 135 kDa laminin-5 subunit (Fig. 4B). In addition, the 155, 135 and 100 kDa species present in MCF-10A matrix co-migrate with the major polypeptides immunoprecipitated from MCF-10A conditioned medium by two laminin-5 monoclonal antibodies (GB3 and C2-9) (Fig. 4C, lanes 1 and 3). The 135 kDa polypeptides immunoprecipitated from MCF-10A conditioned medium by both these anti-laminin-5 monoclonal antibodies are recognized by the β2 chain antibody in immunoblots (Fig. 4C, lanes 2 and 4).

MCF-10A cells undergo branching morphogenesis when plated on matrigel

When MCF-10A cells are embedded into liquid matrigel, which is then allowed to gel, they remain as discrete cellular aggregates ('acini') for 7 days or more regardless of cell concentration (Howlett et al., 1995). In contrast, MCF-10A cells form an interconnected set of tube-like structures, one day after being plated at a concentration of 2.5×10^4 cells/cm^2 on top of matrigel (Fig. 5A). These are similar to the networks of HMECs observed in matrigel and collagen I gels (Bergstraesser and Weitzman, 1996; Berdichevsky et al., 1994).

The ability of MCF-10A cells to assemble into tube-like arrays is cell concentration dependent. At cell concentrations of 1.25×10^4/cm^2 or below the MCF-10A cells remain as small aggregates on the matrigel (Fig. 5B). Indeed, they remain in similar aggregates even at 7 days following plating (result not shown).

The tube-like multicellular aggregates of MCF-10A cells in matrigel were processed for confocal immunofluorescence microscopy using antibodies against laminin-5, α6 integrin and α3 integrin (Fig. 6). Both laminin-5 and α6 integrin are concentrated along the edges of the MCF-10A tubes where the

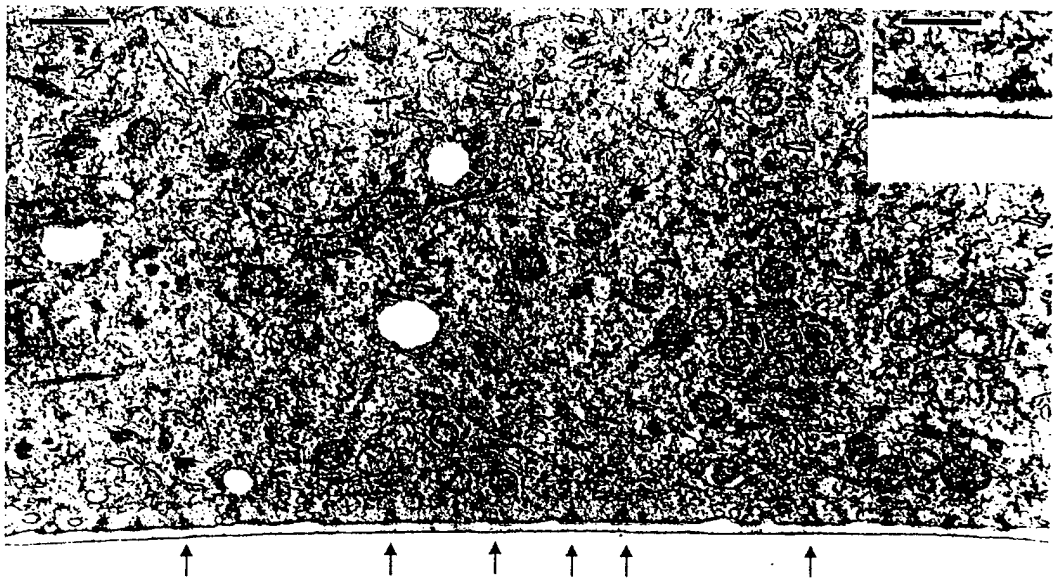


Fig. 3. MCF-10A cells assemble hemidesmosomes when maintained in vitro. This electron micrograph shows a cross section of MCF-10A cells. Arrows indicate numerous electron dense hemidesmosome structures. These possess tripartite cytoplasmic plaques (inset, arrow). Bar, 500 nm (inset, 250 nm).

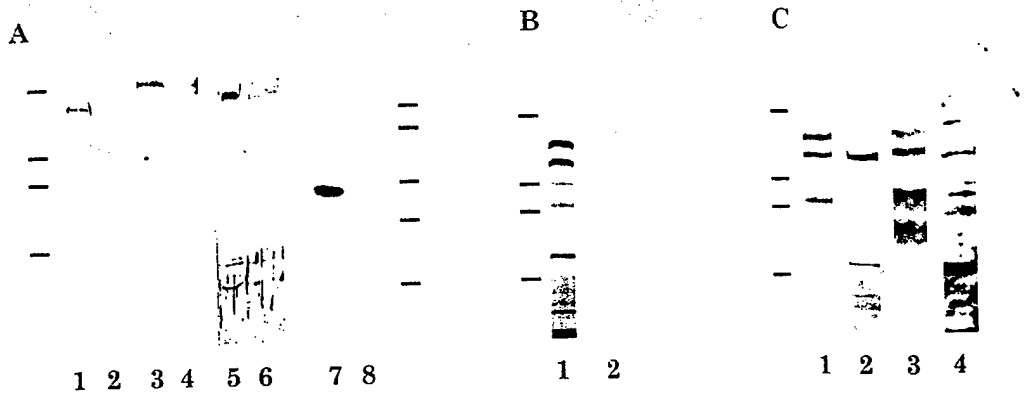


Fig. 4. (A) Hemidesmosomal proteins are expressed by MCF-10A cells as shown by immunoblotting. MCF-10A cell extracts (lanes 1,3,5 and 7) and extracts of SCC12 cells, a keratinocyte line (lanes 2,4,6 and 8) were separated by SDS-PAGE on either 6% (lanes 1-6) or 15% (lanes 7,8) polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with antibodies against BP180 (J17, lanes 1,2), BP230 (10C5, lanes 3,4), β4 integrin (6945, lanes 5,6), or the 'light' chain of α6 integrin (6845, lanes 7,8). (B) MCF-10A cells deposit laminin-5 on their substrate. MCF-10A matrix was collected according to the method of Gospodarowicz (1984), processed for SDS-PAGE on a 6% gel, and either silver stained (lane 1) or transferred to nitrocellulose and immunoblotted with a monoclonal antibody (clone 17) against the β chain of laminin-5 (lane 2). In the silver stained preparation, there are prominent polypeptides at 150, 135 and 100 kDa representing the α, β and the γ chains of laminin-5 (lane 1). The 135 kDa protein in this preparation is recognized by the clone 17 antibody (lane 2). (C) MCF-10A secrete laminin-5 into their medium. The medium conditioned by radio-labeled MCF-10A cells was processed for immunoprecipitation using two monoclonal laminin-5 antibodies (GB3, lanes 1,2; C2-9, lanes 3,4). The immunoprecipitated proteins were analyzed by SDS-PAGE/autoradiography (lanes 1 and 3) or prepared for immunoblotting using clone 17 monoclonal antibody against the β chain of laminin-5 (lanes 2 and 4). The laminin-5 antibodies precipitate three major polypeptides of 150, 135 and 100 kDa (lanes 1,3). The 135 kDa protein is recognized by the clone 17 antibody (lanes 2,4). Note that there is some breakdown of the laminin-5 in the C2-9 antibody precipitate (lane 2). This may explain the ladder of proteins recognized by the clone 17 antibody in lane 4. The low molecular mass reactive species in lanes 2 and 4 are due to cross reactivity of the secondary antibody anti-mouse IgG with the immunprecipitated mouse IgG. Bars on the left side of A, B, and C indicate molecular mass standards of 200, 116, 97.4, and 66 kDa. Bars on the right side of A indicate standards of 66, 45, 31, 21.5 and 14.5 kDa. Each lane of the gels was loaded with approximately 10 μg of protein.

cells abut matrigel (Fig. 6A,B). α3 integrin is localized at the latter sites although it is also present at areas of cell-cell contact (Fig. 6C). An IgG control fails to stain the cell population in Fig. 6D.

Antibody inhibition of MCF-10A morphogenesis

We next used an immunological approach to assess the potential role of laminin-5 and its receptors (the integrins α6β4 and α3β1) in matrigel induced branching morphogenesis of MCF-10A cells. For these studies MCF-10A cells were incubated for 15 minutes at 37 C in medium containing either control IgG (50 μg/ml) or in function blocking antibodies directed against α6 integrin (GoH3 at 50 μg/ml), α3 integrin (P1B5 diluted 1:20) and laminin-5 (C2-9 diluted 1:5)(Fig. 7). The cells in the antibody containing medium were plated onto matrigel coated surfaces at $2.5\times10^4/cm^2$. After 24 hours the cells incubated in control IgG had formed long interconnected tubes whereas there was an obvious inhibition of branching morphogenesis in cultures which had been incubated in the α3 and α6 integrin antibodies as well as those cells incubated with the laminin-5 antibodies (Fig. 7).

We also fixed and processed the antibody treated cells for electron microscopy. We analyzed at least twenty MCF-10A cells in contact with matrigel under each experimental condition (Fig. 8). MCF-10A cells plated onto matrigel in the presence of control IgG assemble hemidesmosomes at sites of cell-matrigel association (Fig. 8A). The latter appear as electron dense structures with extracellular sub-basal dense plates which indicate formation of 'mature' hemidesmosomes (Fig. 8A, inset). In contrast, no hemidesmosomes were observed along regions of cell-matrigel interaction in cultures incubated in function blocking α3 integrin, α6 integrin and laminin-5 antibodies (Fig. 8B-D).

Conclusions

In this study we have shown that MCF-10A cells, an immortalized mammary epithelial cell line, like HMECs, derived from reduction mammoplasties, undergo a branching morphogenesis when maintained on matrigel (Bergstraesser and Weitzman, 1996). This phenomenon is highly dependent on cell concentration. We have never observed the formation of tubular arrays when MCF-10A cells are plated onto matrigel

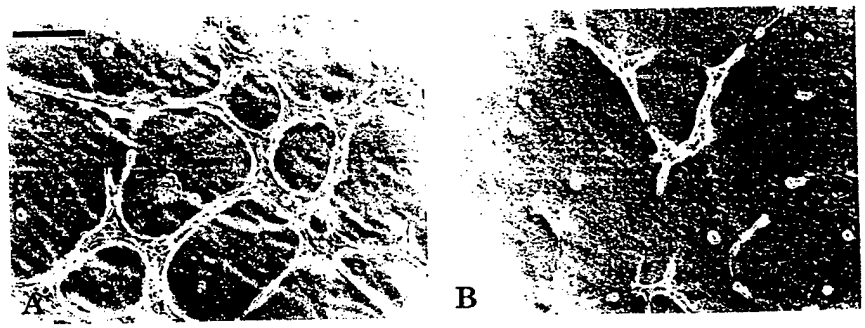


Fig. 5. MCF-10A cells undergo branching morphogenesis on matrigel in a cell concentration dependent manner. $2.5\times10^4/cm^2$ (A) and $1.25\times10^4/cm^2$ (B) MCF-10A cells were plated onto matrigel which had been used to coat 35 mm dishes. At 24 hours following plating, the cells in A have undergone a branching morphogenesis while the cells in B appear in small aggregates. Bar, 500 μm.

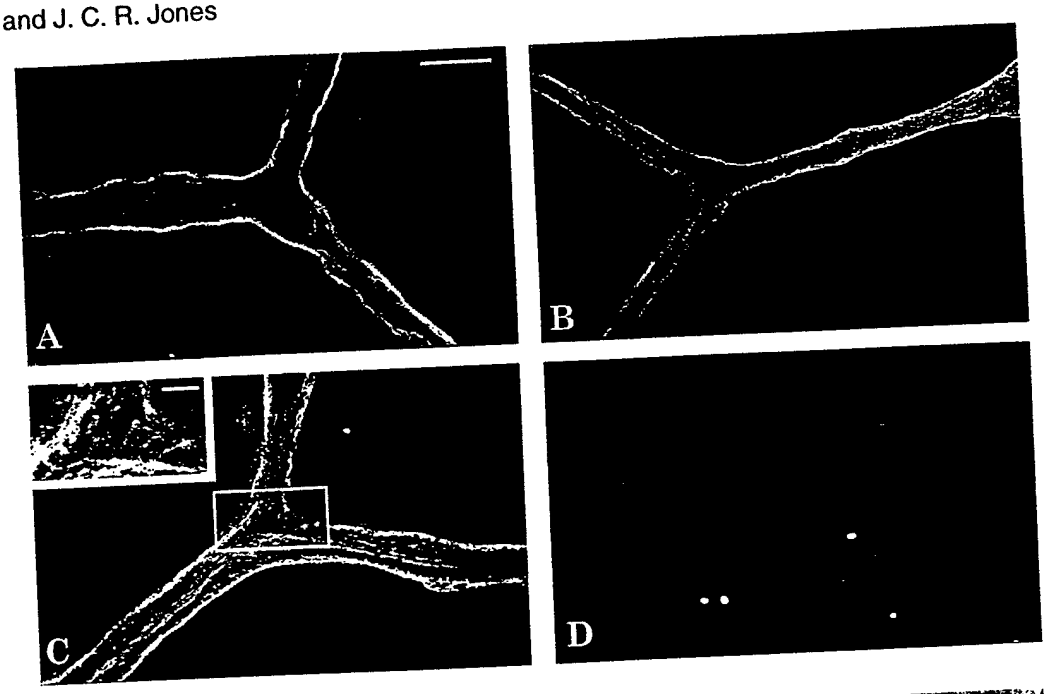


Fig. 6. Laminin-5 and its receptors are expressed by MCF-10A cells undergoing morphogenesis on matrigel. MCF-10A cells maintained in matrigel for 24 hours were processed for indirect confocal immunofluorescence with monoclonal antibodies recognizing laminin-5 (GB3, A), α6 integrin (GoH3, B), α3 integrin (P1B5, C), or an IgG control (D). Note that the antibodies in A,B and C show staining along regions of cell-matrigel interaction. The inset in C is a higher magnification of the boxed area and reveals that α3 integrin occurs at sites of cell-cell as well as cell-matrigel interaction. Bars: (A), 100 μm; (C), 25 μm.

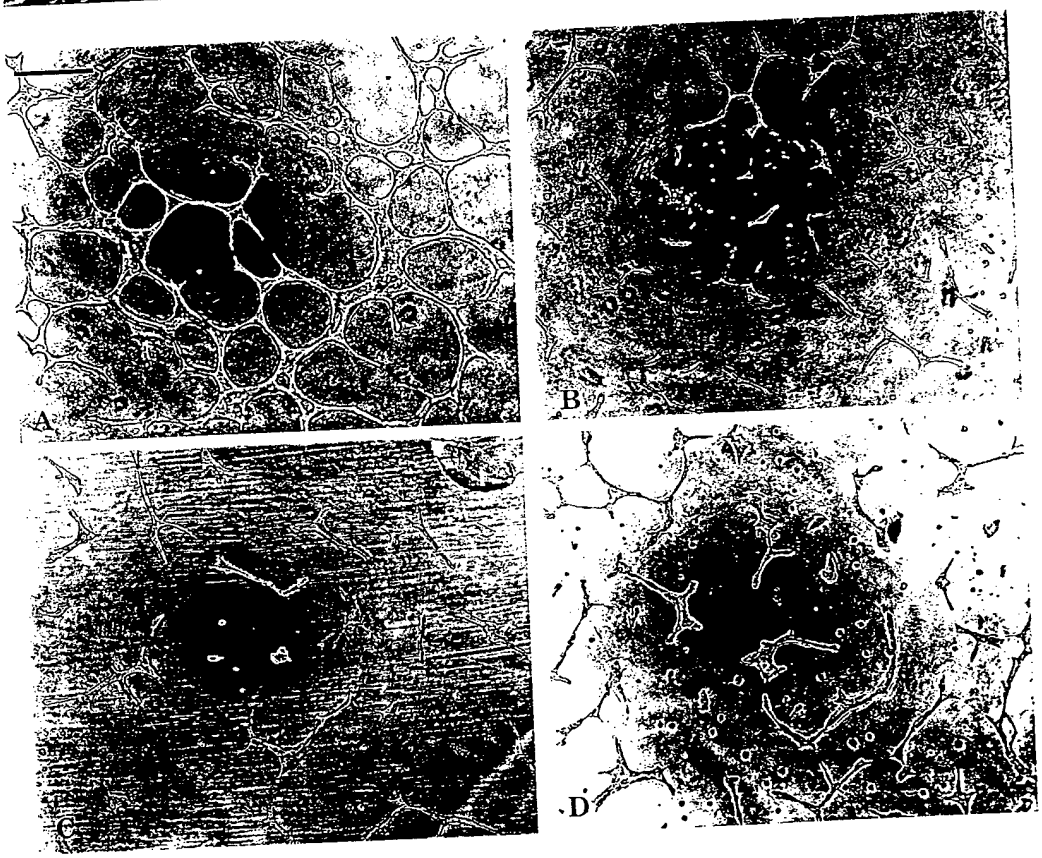


Fig. 7. Branching morphogenesis of MCF-10A cells on matrigel is inhibited by antibodies against laminin-5, α6 integrin and α3 integrin. MCF-10A cells were plated onto matrigel in the presence of control IgG (A), or antibodies which block the function of α6 integrin (GoH3, B), α3 integrin (P1B5, C), or laminin-5 (C2-9, D). After 24 hours, the cells in A appear organized into a highly branched array, while those incubated with blocking antibodies remain either as single cells or in small multicellular clusters (B,C and D). The large dark circle in each of the micrographs is an optical artifact. Bar, 100 μm.

at concentrations below 1.25×10^4cells/cm^2. Just a twofold increase in this cell number is enough to trigger a matrigel induced branching morphogenesis of the MCF-10A cells. Indeed, we find it remarkable that within 1 day of plating onto matrigel, MCF-10A cells assemble into an anastomosing network, organized into a branching pattern much like that seen in vivo in postpubertal mammary glands (Daniel and Silberstein, 1987). This type of pattern has been observed by Berdichevsky et al. (1994) when the human mammary cell line HB-2 is maintained in collagen type I gels.

HMECs assemble hemidesmosomes in vivo (Watson et al., 1988). In vitro they are also capable of forming hemidesmosomes, although this generally takes up to 14 days following plating on tissue culture substrates (Bergstraesser et al., 1995). Like HMECs in vivo, MCF-10A cells express the major components of hemidesmosomes as determined by immunofluo-

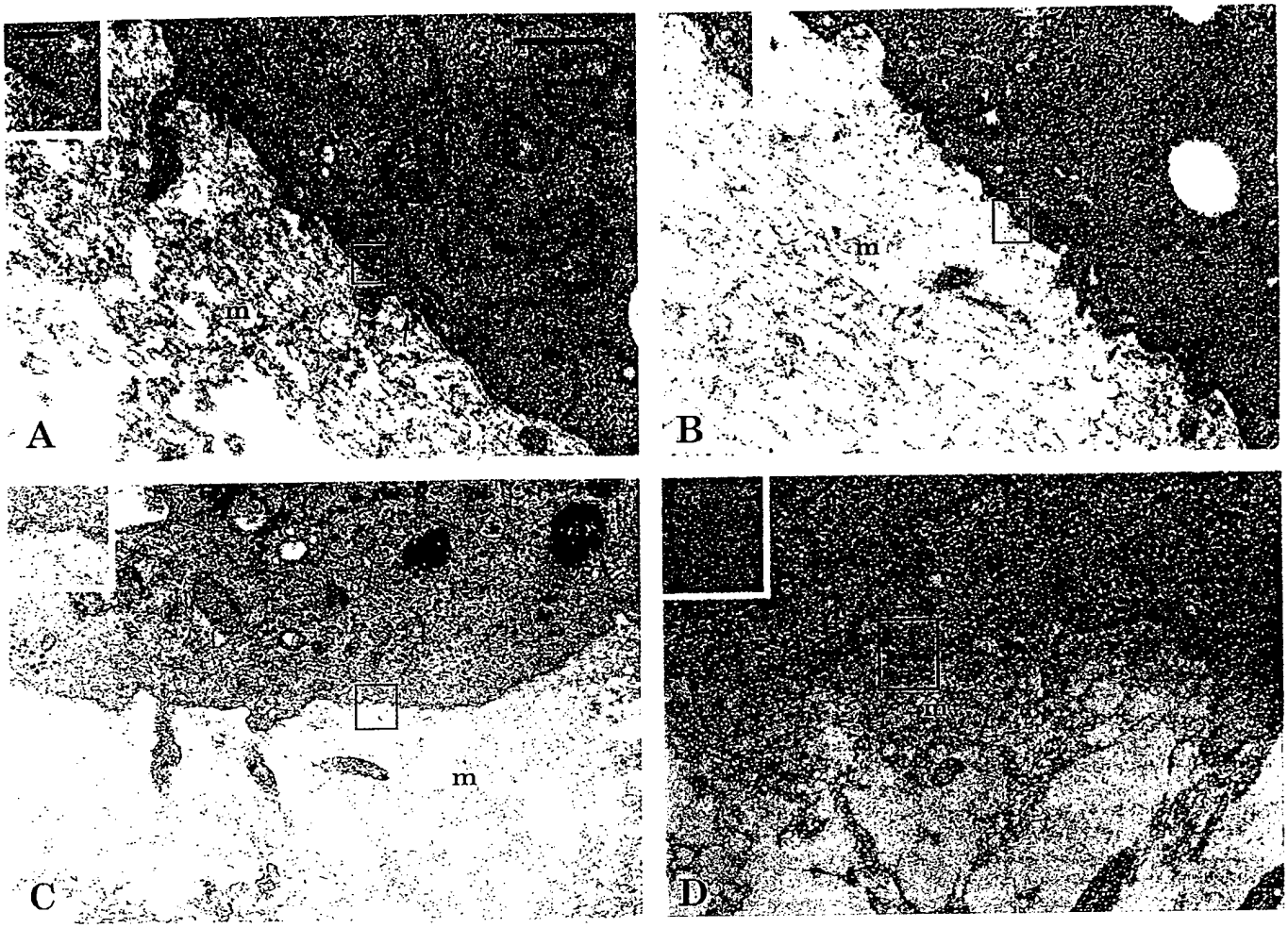


Fig. 8. MCF-10A cells assemble hemidesmosomes on matrigel but this is inhibited by integrin and laminin-5 antibodies. MCF-10A cells were plated onto matrigel in the presence of control IgG (A), or antibodies which block the function of α6 integrin (GoH3, B), α3 integrin (P1B5, C), or laminin-5 (C2-9, D). After 24 hours the cells on matrigel were fixed for electron microscopy. Note that in A there are three hemidesmosome-like structures along the region of cell-matrigel interaction (arrows). One of these (in the box) is shown at higher power in the inset. It possesses an electron dense cytoplasmic plaque and also a sub-basal dense plate. There are no obvious hemidesmosomes in cells in contact with matrigel in B-D (higher power views of these regions are shown in the insets). m, matrigel. Bars: (A), 500 nm; (inset), 60 nm.

rescence, immunoblotting and immunoprecipitation. Moreover, MCF-10A cells readily assemble hemidesmosome-like structures within 24 hours after plating onto uncoated glass coverslips i.e. much faster than their normal counterparts. The speed of hemidesmosome appearance in MCF-10A cells was the more surprising since earlier work had suggested that MCF-10A cells were unable to assemble bona fide hemidesmosomes in vitro (Tait et al., 1990).

When maintained on matrigel, MCF-10A cells assemble hemidesmosomes at sites of cell-matrigel interaction. Consistent with this, a hemidesmosome associated matrix component and its receptor, namely laminin-5 and α6β4 integrin, are distributed at sites of MCF-10A cell-matrigel interaction. Such observations triggered our interest in the potential role of hemidesmosome components in branching morphogenesis of MCF-10A cells. Since it is already established that laminin-5 and α6β4 integrin heterodimer are essential for hemidesmosome assembly, we have been able to assay the role of hemidesmosomes in branching morphogenesis of MCF-10A cells by using antibodies which inhibit both the activities of laminin-5 and α6β4 integrin (Jones et al., 1991; Kurpakus et al., 1991; Spinardi et al., 1995; van der Neut et al., 1996; Georges-Labouesse et al., 1996; Baker et al., 1996).

Function blocking antibodies directed against laminin-5 not only prevent hemidesmosome assembly in MCF-10A cells maintained on matrigel but also significantly inhibit branching morphogenesis. Similarly, antibody GoH3, which blocks α6 integrin function, inhibits both hemidesmosome formation and MCF-10A morphogenesis. Since the α6 integrin subunit is known to preferentially bind β4 integrin in cells which coexpress both of its β1 and β4 integrin partners, as is the case in MCF-10A cells, the inhibitory effects of GoH3 antibodies on MCF-10A cells likely impact the function of the hemidesmosome-associated α6β4 integrin heterodimer (Giancotti et al., 1992; S. E. Baker and J. C. R. Jones, unpublished observations).

Indeed, we assume that matrigel, or more specifically its laminin-1 component, provides an initial framework for MCF-10A attachment and triggers a series of morphogenetic events (Streuli et al., 1995). This includes secretion of laminin-5

which then induces the MCF-10A cells to nucleate the assembly of their own hemidesmosomes, a process requiring laminin-5/α6β4 integrin interaction. We suggest that the formation of the latter complex is necessary to complete branching morphogenesis.

The idea that hemidesmosomes may be involved in morphogenetic events is supported indirectly by recent reports which indicate that hemidesmosomes are sites of signal transduction (Maniero et al., 1995, 1996). For example, the β4 subunit of the α6β4 hemidesmosome associated integrin possesses an unusually long cytoplasmic tail which is associated with one or more protein kinases (Tamura et al., 1990; Maniero et al., 1995). The latter are believed to be involved in a matrix induced cascade of phosphorylation events resulting in phosphorylation not only of the β4 integrin subunit but also of a recently identified protein of 80 kDa (Xia et al., 1996; Maniero et al., 1995).

Laminin-5 and α6 antibodies are not exclusive in their abilities to block morphogenesis of MCF-10A cells in matrigel. A function perturbing α3 integrin antibody, P1B5, is also capable of inhibiting matrigel induced branching morphogenesis of MCF-10A cells. The α3β1 integrin heterodimer is not a component of the hemidesmosome but, like α6β4 integrin is a receptor for laminin-5 (Carter et al., 1990, 1991). In in vitro assays, it has been shown that cell interaction with laminin-5 is initiated by the α3β1 integrin heterodimer (Carter et al., 1991). Subsequently laminin-5 appears to 'switch' receptors and binds to the α6β4 integrin as a prelude to hemidesmosome assembly (Carter et al., 1990, 1991; Spinardi et al., 1995; Xia et al., 1996). Thus one explanation for the morphogenetic impact of the α3 integrin blocking antibody is that P1B5 inhibits the interaction of cells with their own laminin-5. However, we cannot discount that α3 integrin is involved in cell binding to the laminin-1 component of matrigel (Streuli et al., 1995). Of course, P1B5 may inhibit both laminin-1 and laminin-5 interactions of the MCF-10A cells.

In summary, we have identified a model system and a continuous cell line, MCF-10A, for the study of the role of hemidesmosome matrix and integrin components in tissue morphogenesis. In this model, matrigel provides a three-dimensional environment which triggers a series of cellular morphogenetic events, involving the assembly of hemidesmosomes and expression of hemidesmosome matrix and integrin components, in MCF-10A cells. Indeed, it is becoming clear that the hemidesmosome is not simply a spot weld to tether cells to connective tissue but, through the functional properties of its components, the hemidesmosome can have a profound impact on the differentiation and organization of epithelia at the tissue level.

We thank Linda Jaurez for preparing ultrathin sections for electron microscopy and Meredith Gonzalez for technical assistance. We are grateful to Sharon Lang for producing cryosections. This study was supported by the US Army (DAMD17-94-J-4291), the American Cancer Society (CB-69) and the National Institutes of Health (GM38470).

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(Received 16 August 1996 - Accepted 4 October 1996)

Desmosomes and hemidesmosomes: structure and function of molecular components

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ABSTRACT **Desmosomes and hemidesmosomes are the major cell surface attachment sites for intermediate filaments at cell-cell and cell-substrate contacts, respectively. The transmembrane molecules of the desmosome belong to the cadherin family of calcium-dependent adhesion molecules, whereas those in the hemidesmosome include the integrin class of cell matrix receptors. In each junction, the cytoplasmic domains of certain transmembrane junction components contain unusually long carboxy-terminal tails not found in those family members involved in linkage of actin to the cell surface. These domains are thought to be important for the regulation of junction assembly and specific attachment of intermediate filaments via associated adapter proteins. Recent developments have suggested the exciting possibility that these junctions, in addition to playing an important structural function in tissue integrity, are both acceptors and affectors of cell signaling pathways. Many desmosomal and hemidesmosomal constituents are phosphoproteins and in certain cases the function of specific phosphorylation sites in regulating protein-protein interactions is being uncovered. In addition, a more active role in transmitting signals that control morphogenesis during development and possibly even regulate cell growth and differentiation are being defined for cytoplasmic and membrane components of these junctions.—Green, K. J., Jones, J. C. R. Desmosomes and hemidesmosomes: structure and function of molecular components. *FASEB J.* 10, 871–881 (1996)**

Key Words: cell junction · matrix connector · cytoskeleton

STRUCTURE AND DISTRIBUTION OF DESMOSOMES AND HEMIDESMOSOMES

The most prominent cell-surface attachment sites for intermediate filaments (IF)[2] in epithelial cells are desmosomes and hemidesmosomes, which mediate IF anchorage at sites of cell-cell and cell-substrate contact, respectively. By anchoring IF at sites of strong intercellular adhesion, desmosomes create a transcellular network throughout a tissue that is thought to resist forces of mechanical stress. This network in turn is attached to the basal aspect of the cell by molecularly distinct junctional structures called hemidesmosomes, which confer additional mechanical integrity to the tissue. Although providing mechanical integrity is thought to be a critical function of these junctions, it is clear that they are extremely dynamic structures that respond with exquisite sensitivity to environmental cues, allowing for tissue remodeling during development, differentiation, wound healing, and invasion. In addition to being modulated in response to their environment, cell junction molecules themselves play active roles in signal cascades initiated by extracellular matrix ligands and growth factors during development and in the adult.

As their names suggest, desmosomes and hemidesmosomes exhibit similar structural characteristics (**Fig. 1**). Each is composed of a tripartite electron-dense plaque structure specialized for IF anchorage. In the case of the desmosome, mirror image plaques sandwich a membrane core region, whereas a single plaque located at the basement membrane serves this function in the hemidesmosome (insets in Fig. 1). With one known exception, the molecules comprising these junctions are completely distinct, although certain components are evolutionarily related. Extracellularly, desmosomes are separated by a 30 nm space filled with material that represents in large part the extracellular domains of the single span transmembrane desmosomal cadherin molecules. Hemidesmosomes, on the other hand, are attached through an integrin-based mechanism to the underlying basement membrane and stroma.

Although desmosomes and hemidesmosomes are both found in epithelia where they associate with keratin-containing IF, desmosomes are thought to exhibit a more widespread tissue distribution. These intercellular junctions are also present in cardiac muscle where they an-

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[2]Abbreviations: IF, intermediate filaments; BP180, 180 kDa bullous pemphigoid antigen; GABEB, generalized atrophic benign epidermolysis bullosa; CP, cicatricial pemphigoid; JEB, junctional epidermolysis bullosa; IFAP, IF-associated protein; BP, bullous pemphigoid; APC, adenomatous polyposis coli.

0892-6638/96/0010-0871/$01.50.

chor desmin-containing IF, and in the arachnoid and pia of meninges and follicular dendritic cells of the lymphoid system where they associate with vimentin-containing IF (1). In addition to skin and cornea, hemidesmosomes are also present in transitional epithelial cells (e.g., in bladder) and certain glandular epithelia (e.g., mammary gland epithelia and myoepithelial cells) (2, 3). The hemidesmosomes present in all these tissues are related not only by their ultrastructural character, but also by their composition. However, certain hemidesmosomal components also occur in simple epithelial cells such as those lining the gut that lack ultrastructurally defined hemidesmosomes (4–7). In these cases, it has been suggested that hemidesmosomal components are assembled into less organized multiprotein complexes for which some authors have now coined the term type II hemidesmosomes to distinguish them from the "classical" or type I hemidesmsome of basal epidermal cells (7).

Here we present the most recent developments addressing the molecular composition of these two junction types (shown schematically in **Fig. 2**), as well as the structure, function, and regulation of their constituents. This review will not be comprehensive, and we refer the reader to recent review articles for details of other components and a historical perspective of the subject (2, 3, 8).

The desmosome

The membrane molecules

Neighboring cells are thought to be adherent at desmosomes through interactions mediated by a relatively new division of the cadherin family of cell adhesion molecules known as the "desmosomal cadherins." This division includes the subclasses known as desmogleins and desmocollins (reviewed in refs 2, 9). Like the classic cadherins, desmosomal cadherins are single-pass, transmembrane-spanning glycoproteins with conserved regions of homology in the extracellular domain, thought to be involved in calcium binding and adhesion, and a major conserved region in the cytoplasmic domain required for binding to cytoplasmic adapter proteins. In the case of desmosomal cadherins, a protein called plakoglobin associates with this conserved region (reviewed in ref 10). The cytoplasmic domain of the desmogleins also harbors variable numbers of a 29 residue repeating motif of unknown function, unique to this cadherin subclass. Each desmocollin gene gives rise to two alternatively spliced

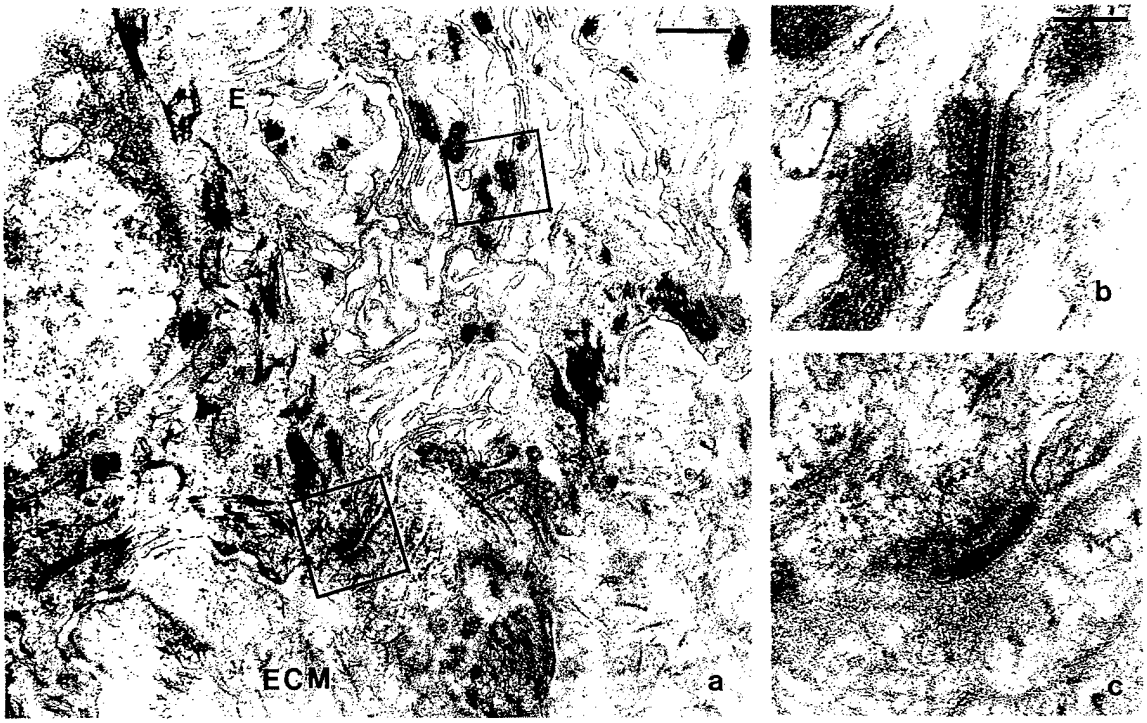

Figure 1. Electron micrographs of the basal layer of human epidermis (E, epidermal cell; ECM, extracellular matrix/dermis). *a);* A region of interaction between two epidermal cells as well as epidermal cell-dermis association is shown. The upper box in panel *a* has been printed at higher magnification in panel *b*. Note the desmosome, with its characteristic electron-dense cytoplasmic plaques, lying either side of the contacting membranes of the epidermal cells. The lower boxed area in panel *a* is printed at higher magnification in panel *c* and shows a typical hemidesmosome. Like the desmosome in panel *b*, the hemidesmosome has a cytoplasmic plaque, but unlike the desmosome, each hemidesmosome abuts the dermis via the basement membrane. Panels *b* and *c* are at the same magnification. *a)* Bar, 500 nm; *b)* bar, 250 nm.

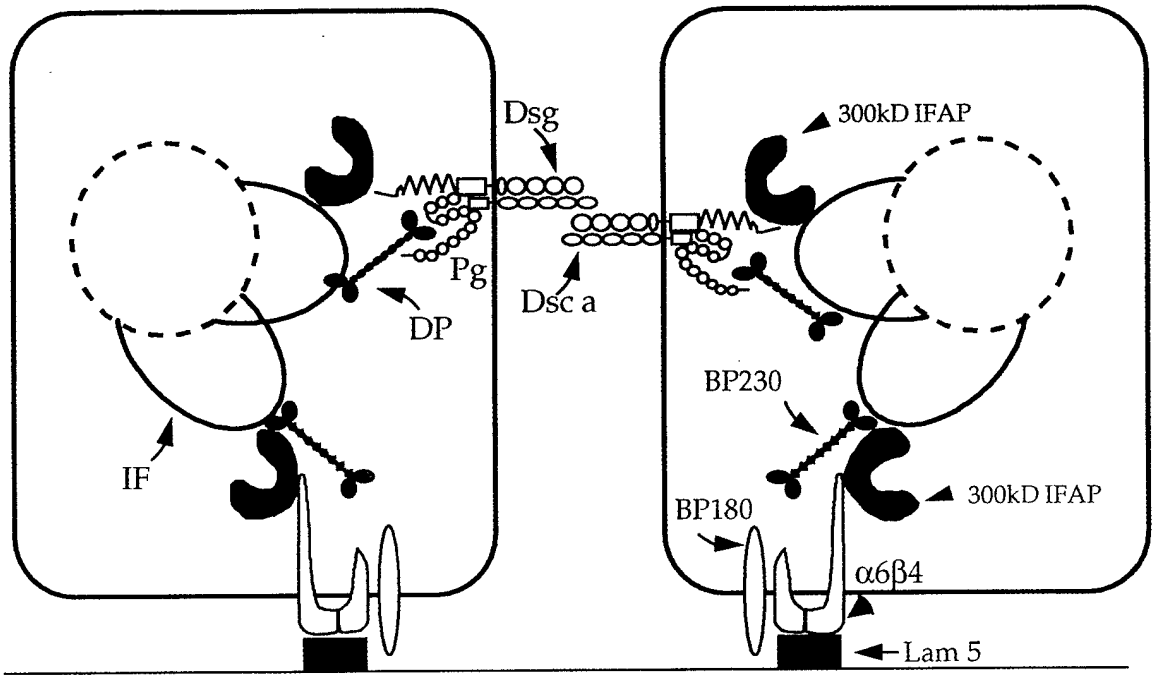

Figure 2. Schematic showing the major components of desmosomes and hemidesmosomes in two epithelial cells. One desmosome links the lateral domains of two epithelial cells while each is tethered to the underlying connective tissue via hemidesmosomes. Both the desmosome and hemidesmosome are connected to the intermediate filament (IF) cytoskeleton system which shows interaction with the surface of the nucleus (central shaded circle). We have taken some liberties in the diagram by indicating our ideas of how proteins may interact within these complex morphological entities. Dsg and Dsc a denote the cadherin-like desmogleins and desmocollins of the desmosome respectively. We only indicate the "long" or a isoform of desmocollin in the diagram. Pg, plakoglobin. desmoplakin, desmoplakin. Lam-5, laminin-5.

mRNA transcripts resulting in an "a" and "b" form, which differ only in the cytoplasmic domain, with the shorter "b" form containing 11 amino acids not in the "a" form. Although the functional significance of these two forms is unknown, the shorter desmocollin tail lacks the plakoglobin binding site present in the longer "a" form and in all desmogleins (11).

The recent identification of three desmocollin and three desmoglein genes has confirmed previous immunological evidence that the desmosomal glycoproteins are heterogeneous and expressed in tissue- and stratification-specific patterns (12–14). The desmosomal cadherins have been mapped to a small cluster on human chromosome 18q21; in the case of the desmogleins, they are tandemly linked in the order DSG1-DSG3-DSG2 from 5′ to 3′ mirroring their expression pattern from suprabasal to basal in stratified human epidermis (15).

Although the classic cadherins are typically thought to mediate calcium-dependent homophilic adhesion, the mechanism by which desmosomal cadherins function is largely unknown. Early work demonstrated that Fab′ antibody fragments against desmocollin inhibit desmosome assembly in MDBK cells (2). In addition, autoantibodies to desmoglein family members circulating in patients with the class of autoimmune epidermal blistering diseases called pemphigus have been demonstrated to be causative in mouse models of the disease, consistent with a role in disrupting intercellular adhesion (e.g., ref 16). Convincing experimental data supporting a role for individual desmosomal cadherins in calcium-dependent homophilic adhesion are lacking, however. A chimeric molecule with the Dsg3 extracellular domain fused to the E-cadherin cytoplasmic domain was shown to engage in weak homophilic adhesion that was not comparable to adhesion mediated by E-cadherin (17). Likewise, full-length desmoglein 1 and desmocollin 2, expressed with or without the associated plakoglobin molecule, are unable to support the level of adhesion mediated by E-cadherin (A. Kowalczyk and Green, unpublished results). The existence of multiple desmosomal cadherins within a single desmosome, as well as multiple tissue specific isoforms, suggests a functional complexity for these molecules not exhibited by their classic cadherin relatives. For instance, it is possible that the active form of desmosomal cadherin is a cell type-specific heterodimer that associates laterally within the junction. Such tissue-specific pairing may endow desmosomes with distinct adhesive and/or cytoskeletal linking roles.

Although the adhesive function of all possible desmosomal cadherin combinations has not yet been tested, the lack of demonstrable adhesion using traditional assays nevertheless highlights the importance of considering the role of other membrane molecules in desmosome function. Recently, a 22 kDa phospholipid-anchored molecule, called the E48 antigen, has been desmonstrated to confer adhesive properties on MOP8 cells (18).

The expression of E48 is restricted to specific tissues, so it is unlikely that this molecule plays a constitutive adhesive function in all tissues. However, the existence of as yet unidentified cell type-specific forms of E48 required for adhesion in other tissues should be considered.

The plaque molecules

The cytoplasmic plaque of desmosomes is complex and exhibits tissue-specific differences in both structure and composition. The constitutive components are plakoglobin and the most abundant component, desmoplakin. Although more minor, sometimes tissue-specific, components surely also play important roles in modulating adhesive or cytoskeletal interactions, emphasis will be placed on recent work dealing with the function and regulation of the major desmosomal components.

Plakoglobin

Plakoglobin belongs to an emerging gene family that also includes β-catenin, the cadherin-associated protein p120, and the tumor suppressor adenomatous polyposis coli (APC). Members of this family share at the core of their structure a series of repeating motifs first found in armadillo, a downstream effector in the wingless signal transduction pathway responsible for the establishment of segmentation polarity in *Drosophila* (19, 20).

As plakoglobin binds tightly to the cytoplasmic domains of both desmosomal cadherins, desmocollin (the larger "a" form) and desmogleins (11, 21–23), it may serve as a molecular link between the outer and inner portions of the desmosomal plaque. Consistent with this idea, deletion of the plakoglobin binding site in desmosomal cadherins abrogates the ability of these molecules to anchor IF at the plaque (11, 24).

Plakoglobin is not restricted to desmosomes but is a common component of adhesive junctions including microfilament associated cell-cell adherens junctions in epithelial and nonepithelial cells (1, 2, 10). This distribution likely reflects plakoblobin's ability to associate not only with the desmosomal cadherins, but also in separate complexes with the classic cadherins, albeit more weakly (25). A potential role for plakoglobin in adherens junctions is not clear. In fact, analysis of cross-linked junctional complexes from MDCK cells suggests that plakoglobin may not even be effectively recruited into Triton-insoluble adherens junctions in cells that have both desmosomes and adherens junctions (26).

In addition to their structural roles in intercellular junctions, members of the armadillo gene family act as signal transducers (19, 20). Armadillo is the most distal component of the signaling pathway mediated by a secreted protein in *Drosophila*, called wingless, which is homologous to the vertebrate Wnt growth factor family. Although, like its vertebrate relatives, armadillo binds to cadherins in cell-surface, adherens-type junctions, evidence suggests that a cytoplasmic rather than junction-associated form of armadillo proteins is active in signaling. Wingless results in the metabolic stabilization and accumulation of cytoplasmic armadillo, which is correlated with a change in its phosphorylation state due to the inactivation of the upstream serine/threonine zeste white kinase. Evidence that such a pool exerts a signaling effect in vertebrates comes from studies desmonstrating that overexpression by microinjection of β-catenin or plakoglobin mRNA into *Xenopus* embryos leads to the duplication of the embryonic axis, resulting in embryos with two heads, notochords, and neural tubes (27, 28). In the case of plakoglobin, this effect was abrogated by coexpression with the desmoglein cytoplasmic domain (28). The latter result suggests that the proper balance between cadherin-bound and unbound pools of plakoglobin/β-catenin is likely to be crucial for proper signaling during development. Along these lines, the extremely rapid degradation of noncadherin associated plakoglobin recently reported in fibroblasts ectopically expressing this protein may represent a general mechanism for controlling the accumulation of armadillo family members (22).

The mechanism by which the armadillo family members actually affect downstream changes in gene expression is unknown. Intriguingly, however, both β-catenin and plakoglobin have been shown to accumulate in the nucleus in overexpression experiments, and some have speculated that interaction of armadillo family members with a nuclear target may be involved in regulation of gene expression (27, 28). The tumor suppressor gene product APC, mutated in patients with the dominantly inherited disease familial adenomatous polyposis, also binds in a cytoplasmic complex with β-catenin or plakoglobin. The functional implications of this association for regulating cytoplasmic levels or signaling activity of these proteins are not known. However, this observation suggests that β-catenin and plakoglobin may be involved in regulating cell growth control in addition to development and differentiation (29, 30).

Plakophilin/band 6

What was previously called "band 6" in enriched preparations of desmosomes isolated from bovine tissues has now been identified as a plakoglobin-like molecule. Unlike plakoglobin, in vitro evidence indicates that this molecule may bind directly to IF polypeptides (31, 32). Band 6/plakophilin exhibits a broader tissue/cell distribution than previously recognized because it is found in the cytoplasm of several cultured lines, including those from simple epithelia. However, it is not a constitutive component of desmosomes and thus is unlikely to be absolutely required for IF anchorage. If plakophilin proves to be a signaling molecule like its armadillo family relatives, this could provide a potential mechanism for differentiation-specific signaling.

Desmoplakin

Although plakoglobin appears to play an important role in establishing contact with the IF cytoskeleton of des-

mosomes, more likely candidates exist for direct association with IF polypeptides. The most abundant and well-studied of these is desmoplakin. Desmoplakin is a large dumbbell-shaped molecule with a central α-helical coiled rod domain flanked by two globular end domains with distinct functions (33). So far, two alternatively spliced forms derived from a single desmoplakin gene have been reported. The smaller desmoplakin II product is more variably expressed, found at lower levels in nonstratified tissues and absent in certain tissues such as the heart.

Molecular mapping studies using transient transfection of constructs encoding specific domains of desmoplakin indicated for the first time that the carboxyl terminus of this molecule contains sequences that govern its association with IF networks (34). These initial observations were borne out by in vitro studies suggesting that this interaction is direct in the case of type II epidermal keratins, which interact with desmoplakin via amino-terminal sequences (35). Transient transfection studies have also mapped sequences required for association with the desmosomal plaque to the amino terminus of desmoplakin (36). Together, these studies suggest that desmoplakin is a functionally modular protein that acts as a molecular linker to anchor IFs at the desmosome; however, these domain mapping studies did not directly test this hypothesis. To address this idea directly, we recently used a dominant negative approach whereby a region of the amino terminus sufficient for localization and, presumably, binding to components of the desmosomal core was moderately overexpressed in stable A431 cell lines. The result was displacement of endogenous desmoplakin from the plaque and loss of IF anchorage, suggesting that desmoplakin is indeed required for this attachment (E. A. Bornslaeger and K. J. Green, unpublished observations).

Like plakoglobin, desmoplakin is a phosphoprotein. Recent evidence suggests that the interaction between the carboxyl terminus of desmoplakin and IF networks is regulated by phosphorylation of a serine residue located in a cAMP-dependent kinase consensus site 23 amino acids from the carboxy-terminal end of desmoplakin (37). This serine is within a region that had been shown to be required for interaction with keratin (but not vimentin) IF networks, and may represent a regulatory site for interaction with specific filament polypeptides (36). One possible function for such a phosphorylation event might be to prevent desmoplakin from becoming sequestered all along IF in the cytoplasm during its recruitment into desmosomes.

Other members of the desmoplakin gene family

BP230/plectin/IFAP300

Desmoplakin belongs to another emerging gene family whose members are involved in the organization or anchorage of IF networks. The first similarity identified was with BP230, a plaque component and candidate IF linker specifically found in hemidesmosomes that will be described in more detail (33). The third member is plectin, a known IF-associated protein (IFAP) with broad tissue distribution reported to be present in desmosomes and hemidesmosomes. Like desmoplakin, plectin's domain functions have been mapped using transient expression experiments, and the carboxy-terminal repeats have been demonstrated to associate with IF networks in cells (38). However, plectin has been shown to bind in vitro to many IF types, including nuclear lamin B as well as microtubule-associated proteins, α-spectrin and fodrin. Thus, this molecule may function as a universal linking protein.

Another potentially closely related protein called IFAP300 has also been demonstrated to be in desmosomes and hemidesmosomes (39). Similar to plectin, IFAP300 binds to cytoplasmic IF networks in fibroblasts in addition to being localized at both junction types in epithelial cells. Data supporting a central role for IFAP300 in IF anchorage will be discussed below. However, the specific roles each individual family member plays within a particular junction remain unknown. IFAP300 and desmoplakin are both IFAPs located in the desmosome, although desmoplakin is present at higher levels. One possibility is that IFAP300 augments interactions mediated by desmoplakin, contributing to the stability of IF interactions in desmosomes. The possible cell type specificity of family members may also be important for mediating interactions with different types of IF networks.

THE HEMIDESMOSOME

The membrane molecules

Hemidesmosome integrins

Integrins are heterodimeric matrix receptors each composed of an α and a β subunit. These receptors not only form part of the link that integrates the extracellular matrix and the cytoskeleton of cells, but also act to transduce signals (40). Until 1990, it was generally believed that cytoskeleton interactions of integrins were limited to the microfilament system of cells. However, in 1990/1991, several groups showed that the epithelial cell integrin α6β4 was concentrated in the hemidesmosome and therefore was spatially associated not with the actin cytoskeleton, but with keratin containing tonofilaments (reviewed in ref 3).

The α6 subunit

The α6 subunit can bind either the β1 or β4 subunit, but when given a choice it preferentially associates with β4 (41). In many epithelial cells, therefore, despite the presence of the β1 subunit, α6 is found exclusively associated with β4 integrin. There are several isoforms of the α6 integrin subunit (4, 6). Each isoform is synthesized as a 150 kDa polypeptide, which is then cleaved into "heavy" and "light" chains that associate via disulfide bonding. The two best-studied α6 isoforms, α6A and α6B, differ in their cytoplasmic domains, thus providing

a possible opportunity for interaction with different cytoplasmic components (6). The α6A isoform has been localized to tissues that possess typical hemidesmosomes (6); however, α6A is also found in gut epithelial cells lacking bona fide hemidesmosomes but that may assemble type II hemidesmosomes (6, 7). Likewise, α6B is found primarily in the kidney and certain epithelial glands where it may occur in a type II hemidesmosome-like structure (6, 7).

The β4 integrin subunit

The β4 integrin subunit is unique among the β integrins so far characterized because of the presence of an extended carboxy-terminal cytoplasmic tail of more than 1000 amino acids (4, 42). Alternative splicing of the β4 message gives rise to two different forms, each containing two type III fibronectin repeat motifs connected by the variable domain, which is also a site of proteolytic cleavage (4, 41). Most investigators in the field assume that the unusual structure of the β subunit cytoplasmic tail explains why α6β4 integrin is the only integrin heterodimer so far identified that is found associated with the IF cytoskeleton. This assumption is based on studies demonstrating that the cytoplasmic tails of subunits of other integrin heterodimers are often involved in anchorage of the actin cytoskeleton via one or more actin cytoskeleton-associated proteins. Indeed, there is now biochemical evidence to support this possibility, because an IF-associated protein IFAP300 that is a component of the hemidesmosome (39) binds β4 integrin in overlay assays (S. E. Baker and J. C. R. Jones, unpublished observations). Thus, IFAP300 may directly link IF to the β4 integrin cytoplasmic tail in much the same way that talin links actin filaments to the cytoplasmic domain of the β1 integrin subunit (40).

Antibodies directed against the external domains of the β4 integrin subunit inhibit hemidesmosome assembly and perturb the structural integrity of formed hemidesmosomes (43). The importance of the β4 integrin in hemidesmosome formation and stability has been confirmed by recent molecular genetic studies by Spinardi and co-workers (44, 45). These authors have made use of 804G cells, one of only a few cell lines that assemble hemidesmosomes in vitro (46). In their initial study they presented evidence that a region of 303 amino acids in the cytoplasmic domain of β4 is necessary for β4 subunit incorporation into hemidesmosomes, whereas the β4 extracellular domain is essential for β4 interaction with the α6 integrin subunit (44). These same workers have showed that overexpression of a tailless β4 integrin, lacking most of the cytoplasmic domain of the wild-type molecule, has a dominant negative effect that leads to perturbation of hemidesmosome organization (45). A mutation in the β4 integrin gene leading to premature termination of message transcription has now been discovered in one patient afflicted with the blistering skin disease junctional epidermolysis bullosa (47). If β4 integrin plays a role in nucleation of hemidesmosome assembly, its absence could explain a key histological feature of this disease, i.e., a decrease in the frequency of hemidesmosomes.

Recent data provide circumstantial support for the possibility that α6β4 integrin is involved in signal transduction. β4 Integrin is physically associated with one or more protein kinases; upon interaction of the α6β4 with its extracellular ligand, β4 becomes phosphorylated on tyrosine (48). Furthermore, a tyrosine phosphorylation site in the cytoplasmic domain of β4 has been shown to be required for its association with other hemidesmosome components (48). This site lies in a tyrosine-based activation motif or TAM consisting of two possible phosphorylatable tyrosine residues, followed by a leucine at position +3 (48). In addition, a separate tyrosine phosphorylation event in β4 appears to trigger binding of the signaling adaptor molecule Shc, which upon phosphorylation recruits the adaptor Grb2 (48). This study suggests that hemidesmosome integrins may mediate signaling events from the matrix to an epithelial cell in a manner similar to other integrin receptors such as the fibronectin receptor α5β1 (reviewed in ref 40).

The 180 kDa bullous pemphigoid antigen (BP180)

Autoantibodies circulating in some patients afflicted with bullous pemphigoid (BP) recognize a 180 kDa hemidesmosomal protein, variously termed BP180 or BPAG2 (bullous pemphigoid antigen 2). The cytoplasmic domain of this type II membrane protein (i.e., its amino terminus is located in the cytoplasm) is separated by a membrane domain from a short extracellular stretch of highly charged amino acids leading to a region containing a series of GLY-X-Y or collagen-like repeats (49, 50). Based on its collagen-like structure, BP180 has been referred to by some investigators as type XVII collagen. It is generally assumed that the collagen extracellular domain is involved in interactions between BP180 and components of the basement membrane, although the nature of such interactions is yet to be defined.

The importance of BP180 for epidermal-connective tissue interactions has been highlighted by the identification of inherited and autoimmune skin diseases that target the BP180 gene or protein. BP180 is missing from the skin of individuals suffering generalized atrophic benign epidermolysis bullosa (GABEB) (51,52). In one case this has been shown to occur because a mutation in the BP180 gene leads to premature transcription termination of the BP180 message, and thus to a lack of BP180 protein in the skin (52). At the electron microscopic level, hemidesmosomes are either missing or present in a rudimentary state in the skin of GABEB patients; presumably this weakens the attachment of epidermal cells to the basement membrane and leads to blistering. BP180 is also a target for pathogenic antibodies in two autoimmune diseases, bullous pemphigoid and herpes gestationis (53). In particular, the perimembrane noncollagenous extracel-

lular domain of BP180 contains an epitope recognized by some (but not all) BP180 autoantibodies (53). Giudice and co-workers (54) have shown that neonatal mice injected with antibodies against this same epitope develop lesions histologically identical to those seen in bullous pemphigoid patients.

A region of 36 amino acids at the amino terminus of BP180 is required for its polarization in the plasma membrane (55). On the other hand, the perimembrane 27 amino acid noncollagenous domain of BP180, the target for pathogenic autoantibodies, appears to be essential for interactions between BP180 and other hemidesmosomal components. With what hemidesmosome element (or elements) does BP180 interact? Using a molecular genetic approach, Hopkinson et al. (55) have provided evidence that BP180 may interact with α6 integrin because BP180 associates morphologically with the α6 integrin subunit regardless of its β partner and α6 antibodies coprecipitate BP180. Indeed, these same workers have speculated that pathogenesis of BP involves autoantibody induced disruption of BP180-α6 integrin interaction leading to perturbation of the structural integrity of the hemidesmosomes. This would parallel the disruption of the hemidesmosomes observed in in tissue explants treated with a function blocking α6 integrin antibody (43).

Matrix molecules

Laminin-5, also referred to as GB3 antigen, epiligrin, and kalinin, is a newly characterized member of a growing family of laminin heterotrimers and is composed of three subunits termed α3, β2, and γ2 (56). Immunoelectron microscopy has revealed that laminin-5 is concentrated in the basement membrane zone immediately underlying each hemidesmosome in stratified squamous epithelial tissues (3). However, note that some if not all of the chains of laminin-5 are expressed by lung epithelial cells that do not possess bona fide hemidesmosomes but that may assemble type II hemidesmosomes (5, 7).

Laminin-5, like other matrix proteins, is promiscuous with regard to its cell receptors (57). For example, in skin cells maintained in vitro, α3β1 appears to initiate cell binding to laminin-5 (58). α6β1 may also act as a laminin-5 receptor in some tissue cultured cells whereas α6β4 integrin is involved in establishment of so-called long term stable anchoring contacts or hemidesmosomes on laminin-5 rich matrices (57–59). The physiological relevance of these laminin-5/integrin interactions is not yet clear because they may not all occur in vivo. However, it is possible that such promiscuity reflects a functional diversity in laminin-5 that in some way is modulated by the nature of its cell-surface associations. This may help explain why a protein involved in formation of stable anchoring contacts, is also found at sites of active cell motility such as the invasion front of colon carcinomas (60). In this regard, the apparent receptor promiscuity of laminin-5 may result from proteolytic processing of its component chains and thus presentation of previously masked receptor binding sites (61). Alternatively, there is now evidence for the existence of laminin-5 chain isoforms that may exhibit distinct receptor specifities (62).

Laminin-5, like BP180, is the target molecule for antibodies in an autoimmune disease (cicatricial pemphigoid, CP) and has been shown to be deficient in the skin of patients with a genetic disease termed junctional epidermolysis bullosa (JEB) (63–67). CP and JEB are characterized by loss of cohesion between an epidermal cell and the basement membrane as well as the absence of hemidesmosomes, suggesting a role for laminin-5 in hemidesmosome assembly. Consistent with this idea, human SCC12 (squamous cell carcinoma) keratinocytes maintained under normal culture conditions fail to form hemidesmosomes. In contrast, SCC12 cells are induced to assemble hemidesmosomes when maintained on a laminin-5 rich matrix secreted by 804G cells (59). Matrix-induced assembly of hemidesmosomes in SCC12 cells involves reorganization of hemidesmosome components, including α6β4 integrin and BP180, along the cell-matrix interface, resulting in a distribution pattern that overlies the laminin-5 components in 804G cell matrix. In other words, 804G matrix contains a structural cue which when transduced to overlying cells, most likely via α6β4 integrin, can trigger hemidesmosome assembly. It will be interesting to address whether the mechanism of hemidesmosome assembly in this system involves a similar signaling pathway involving phosphorylation of β4 integrin as described by Mainiero et al. (48).

The plaque molecules

BP230 and BP230 isoforms

Autoantibodies directed against a 230 kDa plaque component of the hemidesmosome are found in the majority of bullous pemphigoid serum samples (68). Because it was characterized before BP180, it is sometimes called BPAG1, although we shall refer to it as BP230. BP230 localizes within the region of the hemidesmosome plaque to which keratin IF attach (3), providing a first clue that BP230 may be involved in IF cell-surface anchorage. This idea is further supported by the discovery that BP230 belongs to a family of proteins that include desmoplakin and another IF-associated protein called plectin, all possessing striking sequence similarities in their carboxyl terminus, a likely site of binding to IF (33). Indeed, isolated BP230 molecules even bear a superficial resemblance to desmoplakin because both possess a central rod domain with globular ends (69).

Experimental evidence that BP230 plays a role in organizing the IF cytoskeleton comes from studies of mice in which BP230 has been ablated by targeted homologous recombination (70). Hemidesmosomes in the epidermal cells of these mice lack the innermost cytoplasmic region of the hemidesmosomal plaque and exhibit few, if any, associated IFs (70). This is not entirely surprising given the localization of BP230 antigen and speculation that

this protein binds IF in a manner similar to desmoplakin and plectin. However, an unexpected feature of the BP230 knockout mice is that they develop a neuropathy with similar pathological and behavioral features to mice homozygous for the *dystonia musculorum (dt/dt)* mutation. This is not just a coincidence, because the dt allele has been mapped to the same chromosomal location as the gene encoding the BP230 protein (71). Furthermore, sequence analysis of a candidate dt gene ("dystonin") has revealed that it encodes a BP230 isoform ("dystonin") differing in its amino terminus from the BP230 protein expressed in epidermal cells (71). The putative amino-terminal domain of this neural isoform shows sequence similarity to several actin binding proteins including β-spectrin and α-actinin, suggesting that the protein product of the "dystonin" gene may bind both actin-containing microfilaments and IF (71). The possibility that "dystonin" is found in the nervous system equivalent of the hemidesmosome is an intriguing but untested idea.

"Dystonin" is likely to be just one of a family of BP230 isoforms. Hopkinson and Jones (72) have characterized a 280 kDa isoform ("BP280") in a pancreatic carcinoma cell line that is expressed in a variety of cultured epithelial cells and epidermis but apparently is absent from cells of mesenchymal origin (72). Although BP280 lacks the carboxy-terminal IF binding domain, discussed above, it distributes with the filamentous cytoskeleton in certain epithelial cells (72). This observation suggests that more than one domain may be involved in the interaction between the cytoskeleton and BP230 and its isoforms. Such a possibility will need to be tested experimentally, for example, using the type of molecular genetic approach that has proved so successful for identifying functional domains in desmoplakin, plectin, and BP180 (34, 38, 55).

IFAP300/HD1/plectin

As already discussed, BP230 is a strong candidate for an IF-hemidesmosome plaque linker. However, the IF-associated protein IFAP300 has also been identified in the hemidesmosome, in addition to the desmosome (39). Because this protein binds IF in vitro, it could also function to link IF to the cell surface. By analogy to the focal contact, which contains several actin binding proteins, BP230 and IFAP300 could well play complementary roles in IF-hemidesmosome interactions. Indeed, IFAP300 and BP230 may be more than simply functionally related because a partial sequence of IFAP300 indicates that it possesses some sequence similarities to plectin, a member of the same family as BP230 and desmoplakin (39).

IFAP300 was first characterized as a 300 kDa protein associated with the vimentin cytoskeleton of fibroblast cells (73). It is immunologically related, if not identical, to a high molecular weight protein of bovine corneal hemidesmosomes termed HD1, which has also been proposed as an IF-hemidesmosome linker (74; J. C. R. Jones and K. Owaribe, unpublished observations). HD1, like IFAP300, is not restricted to hemidesmosomes but is expressed in numerous epithelial cell types as well as neuronal cells (74). Because HD1 is often coexpressed with α6β4, it has been proposed that HD1/α6β4 complexes define a type II hemidesmosome, as we have already mentioned (7).

ISSUES RISING AND PERSPECTIVES

Significant advances have been made in the identification and characterization of desmosome and hemidesmosome components. In many cases, molecular genetic analysis has allowed functional assignments for individual protein domains. It is likely that state of the art assays such as the yeast two hybrid approach will continue to identify protein-protein interactions in these junctions as well as new components that may be present at substoichiometric levels. In addition, high-resolution structural studies such as that recently reported for the N-cadherin extracellular domain also promise to reveal new information regarding the structural basis of cell adhesion, and ultimately of cytoplasmic interactions (75).

A major challenge will be to define signaling pathways that regulate junction assembly and dissolution. Both desmosome and hemidesmosome assembly involve a spatially and temporally regulated succession of protein-protein interactions. It is generally believed that assembly of junctional plaques is triggered by the lateral association or clustering of transmembrane protein complexes. In the case of the desmosome, this idea is supported by the studies of Troyanovsky (76) demonstrating that clustering of connexin-desmosomal cadherin tail chimeras in the plane of the membrane recruits other plaque components and leads to anchorage of the intermediate filament cytoskeleton. Clustering of hemidesmosomal integrins is also likely to play a role in nucleation of junction assembly as well as recruitment of cytoplasmic components, including IF bundles (45, 59).

What actually leads to clustering of desmosomal cadherins during normal assembly is not well understood. In cultured cell systems, cell contact and calcium lead to a temporal sequence that begins with homophilic adhesion via classic cadherins and proceeds with adherens junction assembly. Desmosome assembly begins shortly thereafter, and although data from different systems sometimes conflict, it appears to depend on a balance in protein kinase and phosphatase activity (see, for example, ref 77). Data from calcium induction experiments contrast with recent evidence demonstrating that half desmosomes can be assembled on their own without a counterpart on an adjacent cell and suggesting that cell contact/calcium serves not as a signal, but simply allows adjacent half desmosomes to interact through the desmosomal cadherin's extracellular domains (78).

Regulating junction dissolution may be equally important, particularly in processes such as wound healing and

invasion. A correlation has been made between growth factor-dependent tyrosine phosphorylation of plakoglobin and a more invasive cell state (79); plakoglobin has also been shown to bind to tyrosine kinase growth factor receptors (80). Thus, in tumors where adhesion is compromised and motility is enhanced, phosphorylation of the catenins or plakoglobin may contribute to loss of cadherin function.

The function of individual proteins, though increasingly well defined in cultured cells, for the most part still needs to be determined at a tissue level. In the case of hemidesmosomes, recently identified inherited diseases are providing important functional information regarding protein components of hemidesmosomes. Although autoimmune diseases that target the desmoglein family of desmosomal molecules are well recognized, so far no genodermatoses have been attributed to mutations in desmosomal components. However, certain genodermatoses have been narrowed down to the desmosomal cadherin chromosome, and within the next 5 years this frontier is sure to be broken (81). In addition, studies in a variety of developmental model systems are beginning to elucidate the function of individual proteins in morphogenesis and differentiation in complex systems and tissues in vivo, and in certain instances are yielding unpredicted and surprising results. For example, gene ablation studies in mice have led to the discovery that a BP230 isoform has unexpected functions in the nervous system (70), whereas studies in flies and frogs have revealed that plakoglobin and its relatives play a central signaling role in embryogenesis (20). Advances in in vitro and cell culture techniques, along with the insights provided by more complex model systems, promise to make the near future a time of great strides in our understanding of cell junctions. FJ

The authors would like to thank all of our colleagues who generously shared their recent published and unpublished work with us during the preparation of this review. Regrettably, we were unable to include citations for all original research due to space constraints, but in such cases have attempted wherever possible to cite reviews covering all the work. Thanks go to A. Kowalczyk for critical reading of the manuscript and to S. Baker for assistance with the figures. Work from the laboratory of K.J.G. is supported by NIH RO1 AR41836, NIH RO1 AR 43380, CB110B, and a basic research grant from the March of Dimes. Work from the laboratory of J.C.R.J. is supported by NIH RO1 GM38470, American Cancer Society CB69, and the U.S. Army DAMD17-94-J-4291. K.J.G. is a recipient of an American Cancer Society Faculty Research Award.

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American Journal of Pathology, Vol. 147, No. 6, December 1995

Expression of Hemidesmosomes and Component Proteins Is Lost by Invasive Breast Cancer Cells

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Hemidesmosomes are multiprotein structures that attach basal cells of stratified epithelia to basement membranes. Although normal human breast epithelia are not stratified, we observed expression of electron-dense hemidesmosomes and hemidesmosome protein components by breast epithelial and myoepithelial cells at the basal lamina* in vivo. *Primary cultured normal human breast epithelial cells also contained hemidesmosomes and component proteins, and could be used as a model for hemidesmosome assembly and regulation. In these cultured cells, hemidesmosome proteins were expressed and localized basally in an unvaried temporal pattern, and electron-dense hemidesmosomes were not seen until the final protein was localized to the cell base. In addition, rate of localization was influenced by confluence, doubling time, and extracellular matrix. Invasive breast cancer cells did not express hemidesmosomes or most of the component proteins* in vivo. *In carcinoma* in situ, *cells away from the basement membrane lacked hemidesmosomes and hemidesmosome proteins, and cells at the basement membrane exhibited abnormalities of hemidesmosome protein expression. Primary human malignant breast cells in culture exhibited a mix of hemidesmosome phenotypes. These data suggest that hemidesmosomes may be important subcellular structures in determining the cytoarchitecture of the breast epithelium. Further, their downregulation may influence cytoarchitecture remodeling closely linked with cell cycle, motility, and extracellular matrix interactions; and their loss in carcinoma may be associated with loss of normal cytoarchitecture. (Am J Pathol 1995, 147:1823–1839)

A body of literature has been accumulating suggesting that adhesion molecules, those proteins and other substances that cells use to adhere to their substrate, may play a role in cancer cell invasion and metastasis.[1, 2] Data have suggested on the one hand that cancer cells may exhibit reduced adhesion molecule expression or function resulting in release of their substrate, and freeing cells to pile up or migrate. On the other hand, carcinoma cells may acquire expression of new adhesion molecules to grip the basement membrane (BM) in order to traverse it, or to adhere to tissues at sites of metastasis.[3, 4]

We and others have previously addressed this subject in the breast in studies of the integrin class of cell adhesion molecules, several of which are present in normal breast tissue, but reduced or absent in carcinoma (see, e.g., refs. 5–9). Hemidesmosomes (HD) are another adhesion structure to study in this context. They are found only in epithelial cells,[10] which are usually stationary, but not in cells such as fibroblasts or macrophages, which wander (for recent reviews see refs. 11–13). Further, HD, more than other adhesion structures, may mediate firm, relatively immobile attachment to the BM,[14, 15] preventing the cell movement characteristic of invading malignant cells.

HD loss has been seen in cutaneous basal cell carcinoma.[16, 17] In addition, the $\alpha 6\beta 4$ integrin is a

Supported by US Army grant DAMD 17–94-J-4291, The Lynn Sage Foundation, the Cooperative Human Tissue Network, which is funded by the National Cancer Institute, the American Cancer Society (CB-69), and the NIH (GM38470).

Accepted for publication August 8, 1995.

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component of HD[18–20], and we and others[5–7, 21, 22] found that the $\alpha 6$ and $\beta 4$ integrin subunits were not expressed in some malignant mammary epithelial cells.

Although HD have been most commonly described in stratified epithelia, and the breast parenchyma is not normally stratified, ductal epithelia from various sources have been shown to contain HD.[13] It is thought that epithelia that contain HD may be those under greater shear stress. The breast contains a ductal epithelium that undergoes great shear stress during lactation, and some electron microscope (EM) studies have suggested that at least breast myoepithelial cells, and perhaps luminal cells that contact the BM, may have HD.[23–28] It is not clear, however, whether these electron-dense structures contain the same molecular components described in other well studied HD.

For this paper we studied normal and malignant breast cells in tissue sections and in culture for the presence of HD and some of their constituent and associated proteins including the M_r 180 and 230 bullous pemphigoid antigens, an M_r 200 protein, and collagen VII. Normal human mammary epithelial cells (HMEC) had HD *in vivo* and in culture, and expressed the expected spectrum of HD proteins, whereas invasive carcinoma cells lacked HD *in vivo*, and malignant cells in culture exhibited defects in HD assembly. In addition we used normal HMEC in culture as a model to study HD assembly and regulation.

Materials and Methods

Tissues

Breast tissues were obtained from Northwestern Memorial Hospital, Evanston Hospital, or the Cooperative Human Tissue Network of the National Cancer Institute. All malignant tissues used were infiltrating ductal carcinoma varying from grades I to III, and were from patients with from 0 positive lymph nodes to 10/10 positive level III nodes. Normal breast tissue was derived from such cancer patients, and from reduction mammoplasties. In addition, one sample of normal lactating tissue was obtained as the "normal" tissue from one of the mastectomies. Specimens were obtained fresh from surgery, and processed for EM, frozen sections, or cell culture.

Cell Culture

Mammary epithelial cells were derived from 11 infiltrating ductal carcinomas, 7 reduction mammoplasties, and sites distant from 2 of the carcinomas. Epithelial or carcinoma cells were culled and grown on plastic by the method of Stampfer[29] with revisions for tumor cell growth[30] in MCDB-170 medium (American Bioorganics, Inc., Niagara Falls, NY) plus serum-free supplements.[29]

Mammary epithelial cell strains were determined to be epithelial by their expression of desmosome proteins by immunofluorescence (IF) and determined to be malignant by their ability to proliferate in the absence of certain growth factors, in the presence of transforming growth factor-β, and at high cell concentrations, and their inability to form three-dimensional structures on Matrigel BM-like substance.[30]

804G cells (a rat urinary bladder carcinoma cell line) were maintained in culture as previously described.[31] These cells were utilized for their capacity to produce laminin-5-rich extracellular matrix (ECM), as described below.

Immunofluorescence

Tissues fresh from surgery were snap-frozen in liquid nitrogen and stored at −70°C until use. Pieces of frozen tissue were embedded in Tissue-Tek OCT compound (Miles Laboratories, Elkhart, IN), sectioned on a Tissue-Tek cryostat (Miles Laboratories) to a depth of ≈8 μm, and placed on poly-L-lysine (Sigma Chemical Co., St. Louis, MO)-coated glass microscope slides (VWR, Media, PA). Sections were fixed for 5 minutes in −20°C acetone (Mallinckrodt, Paris, KY) and air-dried. Tissues from seven infiltrating ductal carcinomas, seven normal specimens, and one sample of normal lactating tissue from a cancerous breast were used.

Cultured cells were grown on glass coverslips (VWR) in six-well plates (Falcon, Lincoln Park, NJ) at 10^4 to 5×10^4 cells per well. After three washes in phosphate-buffered saline (PBS) pH 7.4 containing 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 8.0 g/L NaCl, 1.15 g/L Na_2HPO_4, cells on coverslips were fixed for 3 minutes in −20°C methanol (Fisher Scientific, Fair Lawn, NJ) and air-dried. Cells from six carcinomas and four normal tissues (three reduction mammoplasties and one mastectomy) were used.

IF was performed as previously described.[5] Slides were observed and photographed using a Leitz Laborlux D fluorescence microscope and TMAX 100 film (Eastman Kodak Co., Rochester, NY). All photographic exposures were for 1.5 minutes.

The following primary antibodies were used: serum from a bullous pemphigoid patient containing human autoantibodies reactive primarily with an M_r

230 plaque protein of the HD,[32] 180 mouse monoclonal[33] and J17 rabbit polyclonal[34] antibodies to an M_r 180 transmembrane HD protein, 6A5 mouse monoclonal antibody to an M_r 200 HD protein,[35] 9C3 mouse monoclonal antibody, and EBA human autoantibody[36] to collagen VII anchoring fibril protein (which is also the epidermolysis bullosa aquisita antigen). Secondary antibodies included fluorescein-conjugated anti-mouse IgG + IgM, anti-human IgG + IgM and anti-human IgM; and rhodamine-conjugated anti-mouse IgG + IgM and anti-rabbit IgG (Kierkegaard and Perry, Gaithersburg, MD). Antibody concentrations were determined on the basis of concentration curves.

EM

EM was performed using standard methodology.[37] Briefly, 1 mm^3 tissues fresh from surgery were fixed in 2.5% glutaraldehyde in 0.1 mol/L Sorensens's phosphate buffer, postfixed in 1% OsO_4, stained in 2.5% uranyl acetate, dehydrated and infiltrated with propylene oxide, and embedded in Spurr epoxy resin (all from Electron Microscopy Sciences, Fort Washington, PA). Thick sections were examined by a pathologist to confirm diagnoses and find regions of carcinoma. A total of 12 carcinomas and 14 normal tissues (2 from reduction mammoplasties and 12 from cancer patients) were used for EM.

Cultured cells were grown on glass coverslips or on Matrigel (Collaborative Research, Bedford, MA)-covered glass coverslips in six-well plates at 10^4 to 5×10^4 cells/well, processed similarly to tissue samples and embedded in Epon-araldite resin 812 (Tousimis, Rockville, MD, or Fisher Scientific). Samples from five malignant cell strains and five normal cell strains (cells from four reduction mammoplasty patients, and normal cells from one cancer patient) were processed.

Thin sections were cut on a Reichert Ultracut E microtome (Reichert Instruments, Buffalo, NY), mounted on copper grids, stained with uranyl acetate and lead citrate (Electron Microscopy Sciences) and viewed at 80 kV in a JEOL 100CX electron microscope (JEOL USA, Peabody, MA).

Growth of Cells on Matrigel BM-Like Substance

35 mm^2 wells were coated with 500 μl undiluted cold Matrigel, then placed at 37°C for at least 30 minutes. Matrigel, a substance produced *in vivo* by the Engelbreth-Holm-Swarm murine tumor, resembles the lamina lucida portion of the BM, and contains (among many substances) mainly laminin, collagen IV, and proteoglycans.

Preparation of Laminin-5-Rich Matrices from 804G Cells

804G matrix was prepared as described previously.[31] Briefly, 804G cells were grown to confluence on glass coverslips, the culture medium was aspirated, and the cells were washed in sterile PBS and removed from their matrix with 20 mmol/L NH_4OH for 5 minutes followed by three PBS washes. HMEC were plated on the matrix in their usual medium. After 24 hours, HMEC on 804G matrix were fixed in 2.5% glutaraldehyde and processed for EM as above.

Results

In Vivo *(Tissues)*

Mammary epithelial cells *in vivo* contained electron dense HD at their basal plasma membranes where they were in contact with the BM (Figure 1). These triangular-shaped plaques were associated with intermediate filaments intracellularly, and with anchoring filaments and anchoring fibrils extracellularly. HD were seen in all cells where contact with the BM could be shown. This included luminal cells that reached from the lumen to the BM (Figure 1, D and E) and myoepithelial cells that did not appear to reach the lumen (Figure 1B).

In all intraductal regions of malignant tumors, cells in contact with the BM also exhibited apparently normal HD (Figure 2). Some of these cells may have been normal myoepithelial cells, but many had morphological features of malignant cells and lacked the contractile filaments, large numbers of mitochondria, and shape typical of myoepithelial cells. All cells in contact with the BM had HD, whereas cells not at the BM contained no HD, although numerous desmosomes and some adherens junctions were seen (Figure 2).

In all invasive regions examined, no HD were seen in any cells (Figure 3) whether single cells, in small groups, or invading en masse and attached to one another with desmosomes.

Because HD have not been well studied in the breast, we looked by IF microscopy at expression of several of the protein components of HD previously described in skin. The anchoring fibril protein collagen VII (Figure 4C), M_r 180 (Figure 5A), and M_r 230 bullous pemphigoid antigens, M_r 200 protein (Figure 6C), and the α6 and β4 integrin subunits[5] were all

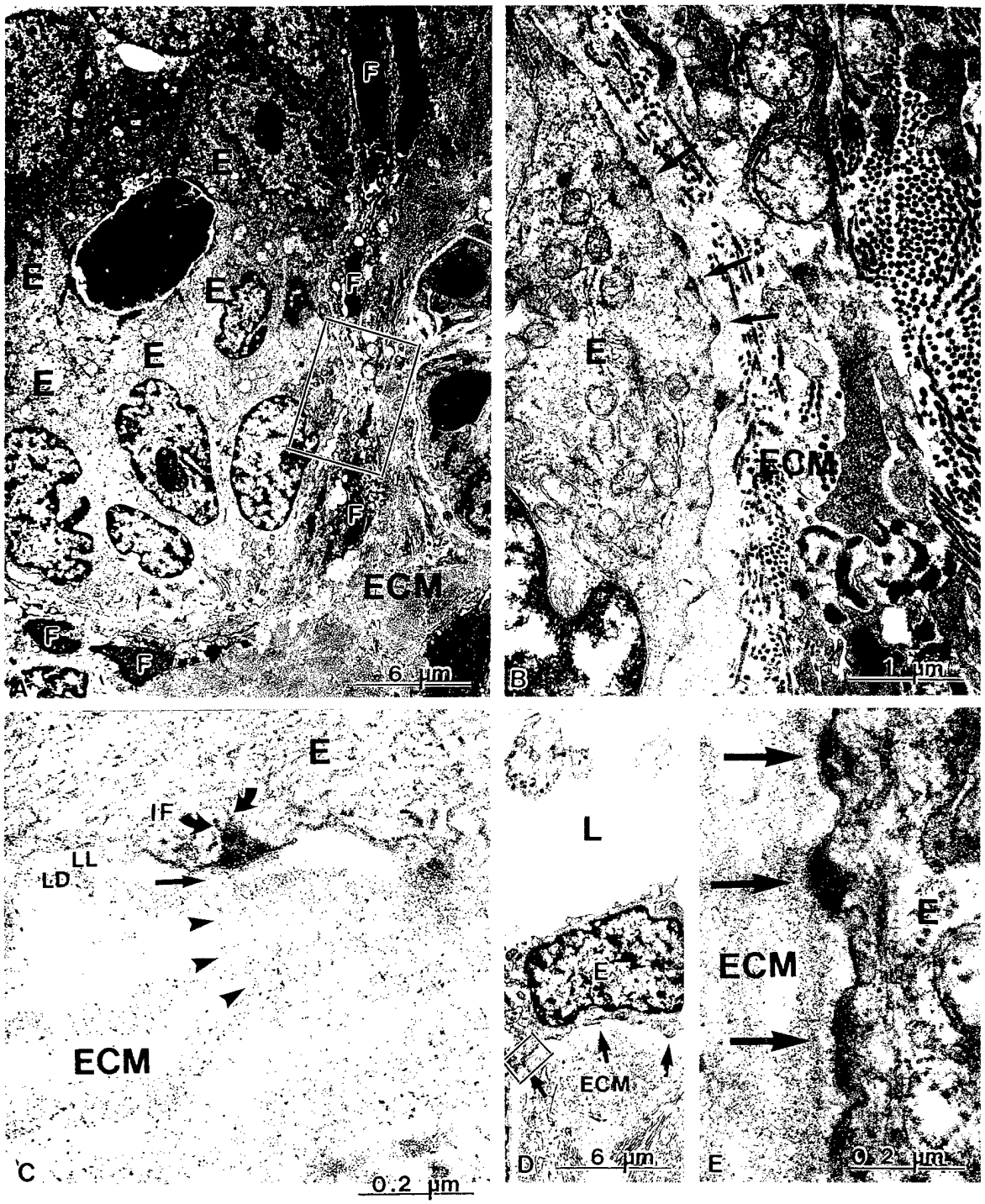

Figure 1. *The presence of hemidesmosomes in normal breast epithelium by electron microscopy.* (A) *A normal duct in cross section.* (B) *Higher magnification of box in* A *shows HD* (arrows) *at the bases of ductal epithelial cells.* (C) *Higher magnification of an HD showing intermediate filaments* (*IF,* curved arrows), *anchoring filaments* (arrow) *and anchoring fibrils* (arrowheads *along the length*). *LL, lamina lucida; LD, lamina densa.* (D) *A luminal cell reaching from lumen to basement membrane containing basal HD* (arrows). (E) *Higher magnification of the box in* D *showing HD* (arrows). *L, lumen;* E, *epithelial cell; F, fibroblast.*

present in normal ducts at the basal aspects of cells (β4 and the M_r 200 protein exhibited a basolateral distribution).

We next wished to determine whether abnormalities of HD protein expression would be seen in breast carcinoma. Two patterns emerged. The first

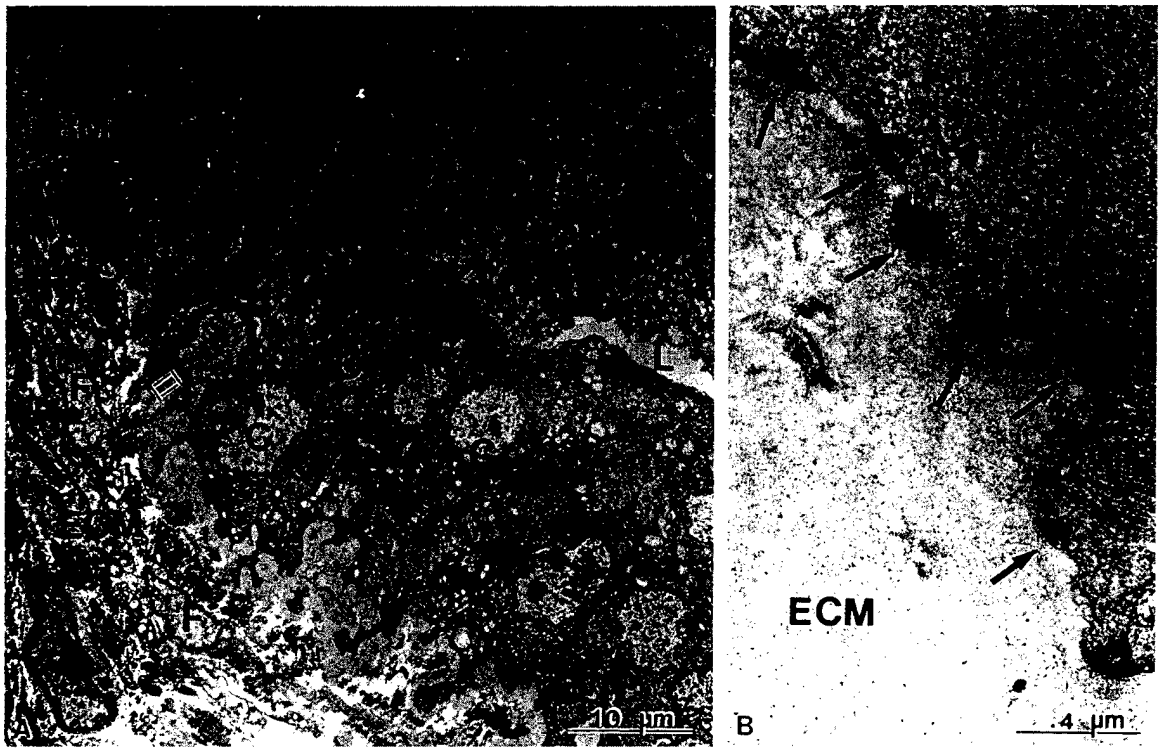

Figure 2. *Presence of HD in breast carcinoma* in situ *by EM.* (A) *An intraductal carcinoma showing many layers of cells within a duct with very little lumen (L) but surrounded by a BM.* (B) *Higher magnification of the box in* A *shows multiple HD* (arrows). *C, carcinoma cell; F, fibroblast.*

pattern, seen with collagen VII, the M_r 180 and 230 proteins and the α6 integrin subunit[5] is illustrated for collagen VII in Figure 4. Staining for the HD proteins was seen at the BM in normal ducts and in intraductal carcinoma. Cells piled up within cancerous ducts, and invasive cells exhibited no staining. A slight variation of this pattern was seen with the M_r 180 protein from which staining in all but one patient exhibited the aforementioned pattern, whereas the malignant cells from one patient exhibited no staining for the M_r 180 protein, even in regions of intraductal carcinoma (Figure 5, C and E).

The second pattern, seen with the M_r 200 protein and the β4 integrin subunit,[5] is illustrated in Figure 6. Staining was seen at the BM in normal ducts. In both carcinoma *in situ* and invasive carcinoma, regions of staining were seen and regions devoid of staining were also observed. For any one patient there were regions of intraductal carcinoma outlined by staining, and intraductal carcinoma without staining, and invasive cells that stained or did not stain.

Cultured Cells

Because we saw abnormalities of HD and HD protein expression *in vivo*, we decided to study cultured HMEC as a prelude to use of these cells for experimental manipulations.

Normal HMEC in culture exhibited electron-dense, apparently normal, HD by 2 weeks in culture (Figure 7). Malignant cell strains varied in their phenotype. For three patients, no cells could be found with HD; for one patient all cells had abundant, apparently normal HD; and the cells of one patient were a mix of cells with no HD, abundant HD, and a few HD.

We next stained cultured HMEC to see if the electron-dense HD seen by EM might also contain the expected protein components. Normal HMEC expressed the anchoring fibril protein collagen VII in a basal secreted pattern (Figure 8), and the M_r 200, 230, and 180 proteins in rows of basal tick-mark-shaped plaques (Figures 9 and 10). The α6 and β4 integrins are also expressed in this manner and colocalize with one another.[5]

Malignant HMEC also expressed all of the HD-associated proteins tested for. However, several abnormalities of expression were consistently noted. When malignant cells were plated on coverslips, it took 2 to 3 weeks to see a basal secreted pattern of collagen VII staining, whereas normal cells showed this pattern in less than a week. In time course experiments (Figure 8) we found that within 1 day normal HMEC produced

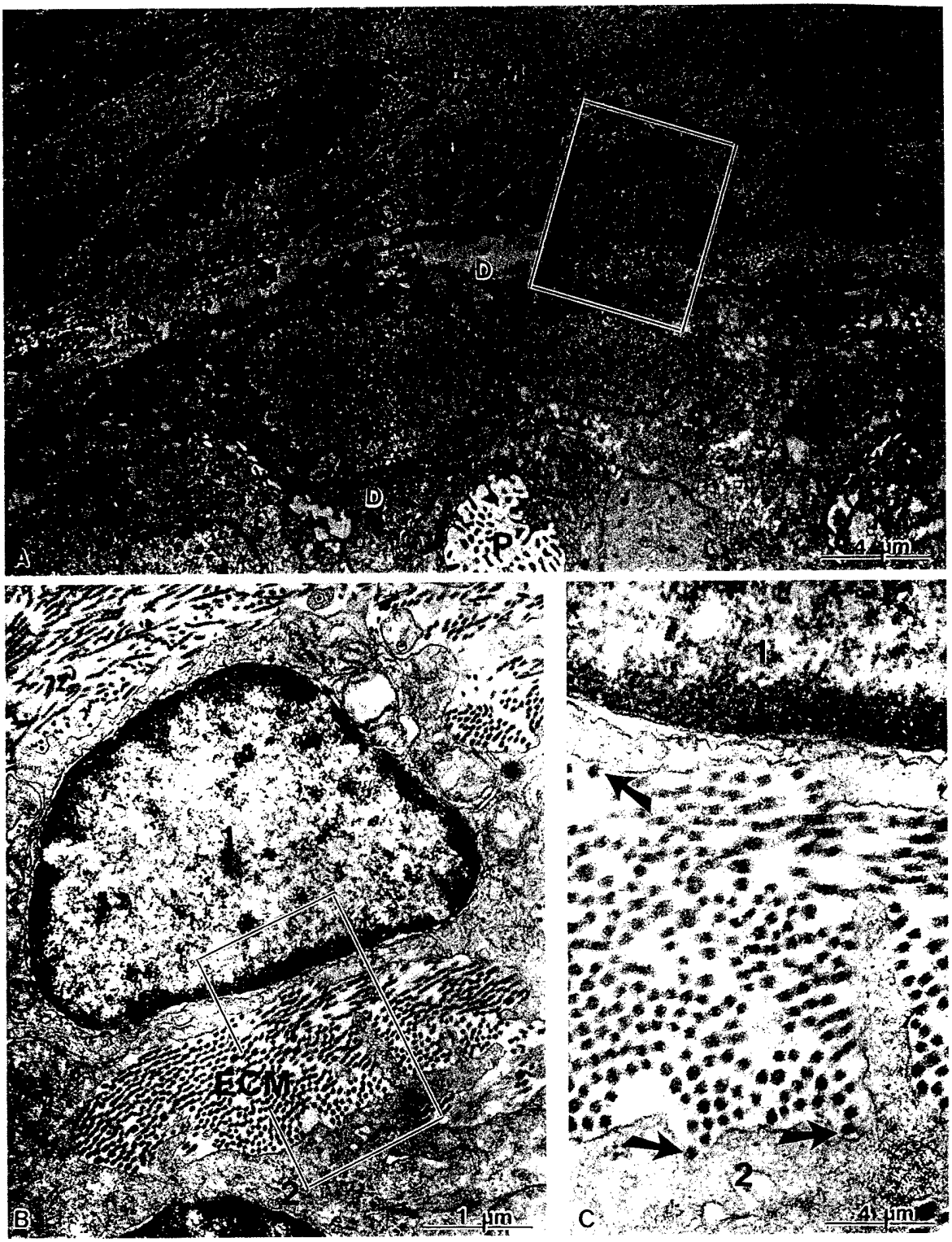

Figure 3. *Absence of HD in invasive breast carcinoma.* (A) *A group of invasive cells is pictured. Note the absence of a BM at the epithelial: ECM border* (curved arrows). *presence of desmosomes* (D, straight arrows). *A cell, apparently at the invasive front, is surrounded by ECM on three sides* (box). *C, carcinoma cell; F, fibroblast; P, pseudolumen.* (B) *Higher magnification of the box in* A *shows two invasive cells (1 and 2) abutting the ECM without any HD.* (C) *Higher magnification of box in* B *shows cells (1 and 2) with cell membranes in direct contact with collagen* (arrows) *without any intervening BM or HD.*

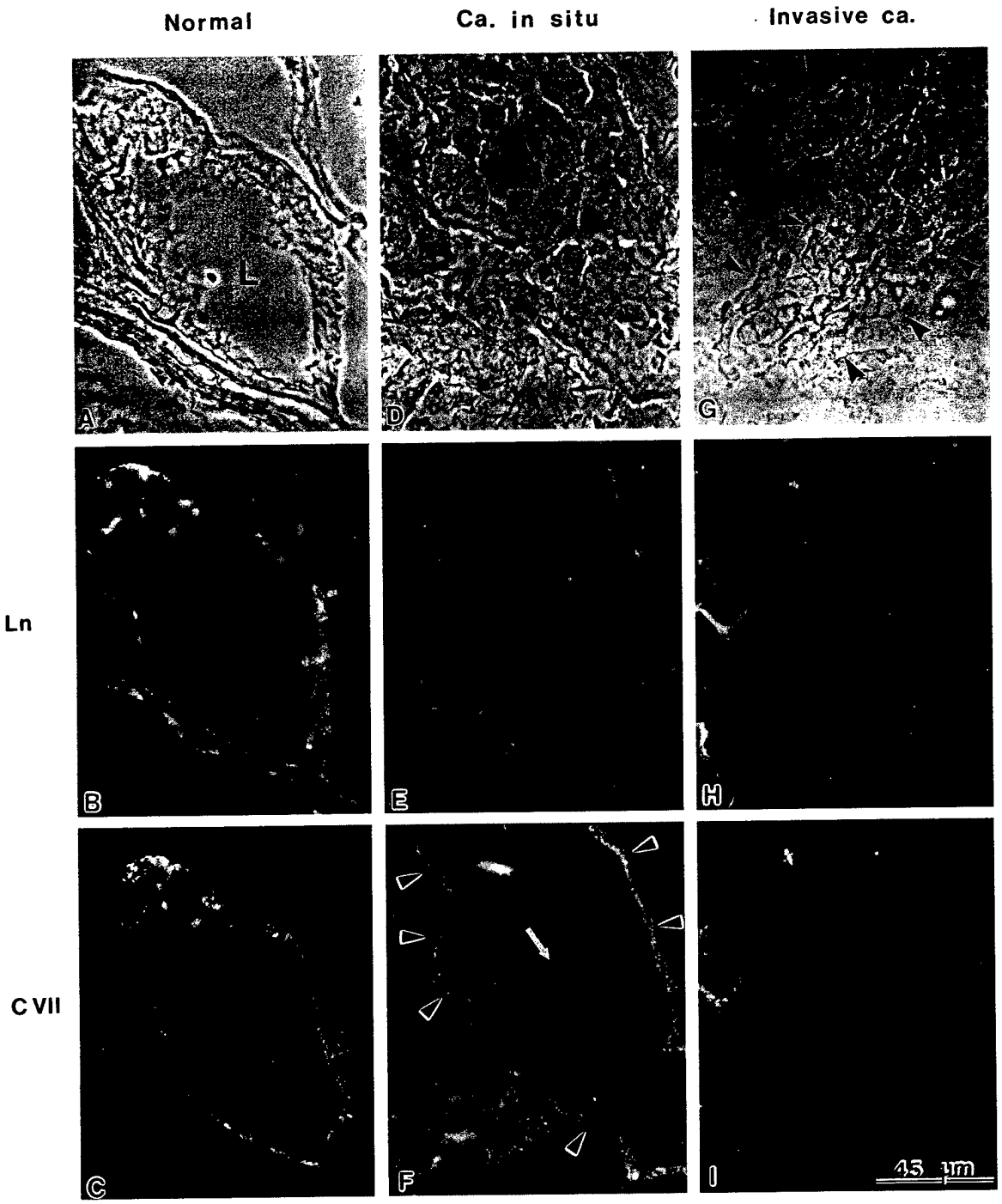

Figure 4. *Expression of the anchoring fibril protein, collagen VII in normal duct and carcinoma* in situ, *but not invasive carcinoma by immunofluorescence.* (A–C) *A normal duct in cross-section* (A) *has a BM as determined by laminin staining* (B) *and expresses basal collagen VII* (C). (D–F) *An intraductal carcinoma* (D) *has a BM around the entire group of cells* (E) *and exhibits collagen VII staining* (F) *only at the basement membrane* (arrowheads), *whereas cells piled up within the duct do not exhibit collagen VII staining* (arrow, *compare with* D, arrow). (G–I) *Invasive carcinoma* (G), *outlined by arrowheads, does not have a BM* (H) *or express collagen VII* (I). *Ln, laminin; C VII, collagen VII; L, lumen.*

antibody-detectable perinuclear intracellular collagen VII, whereas malignant cells had no immunostaining. At about 7 days, malignant cells began to show perinuclear intracellular collagen VII staining, whereas normal cells were already producing basal collagen VII in a secreted pattern.

The M_r 200 protein, on the other hand, continued to be expressed by malignant cells in only a dotted

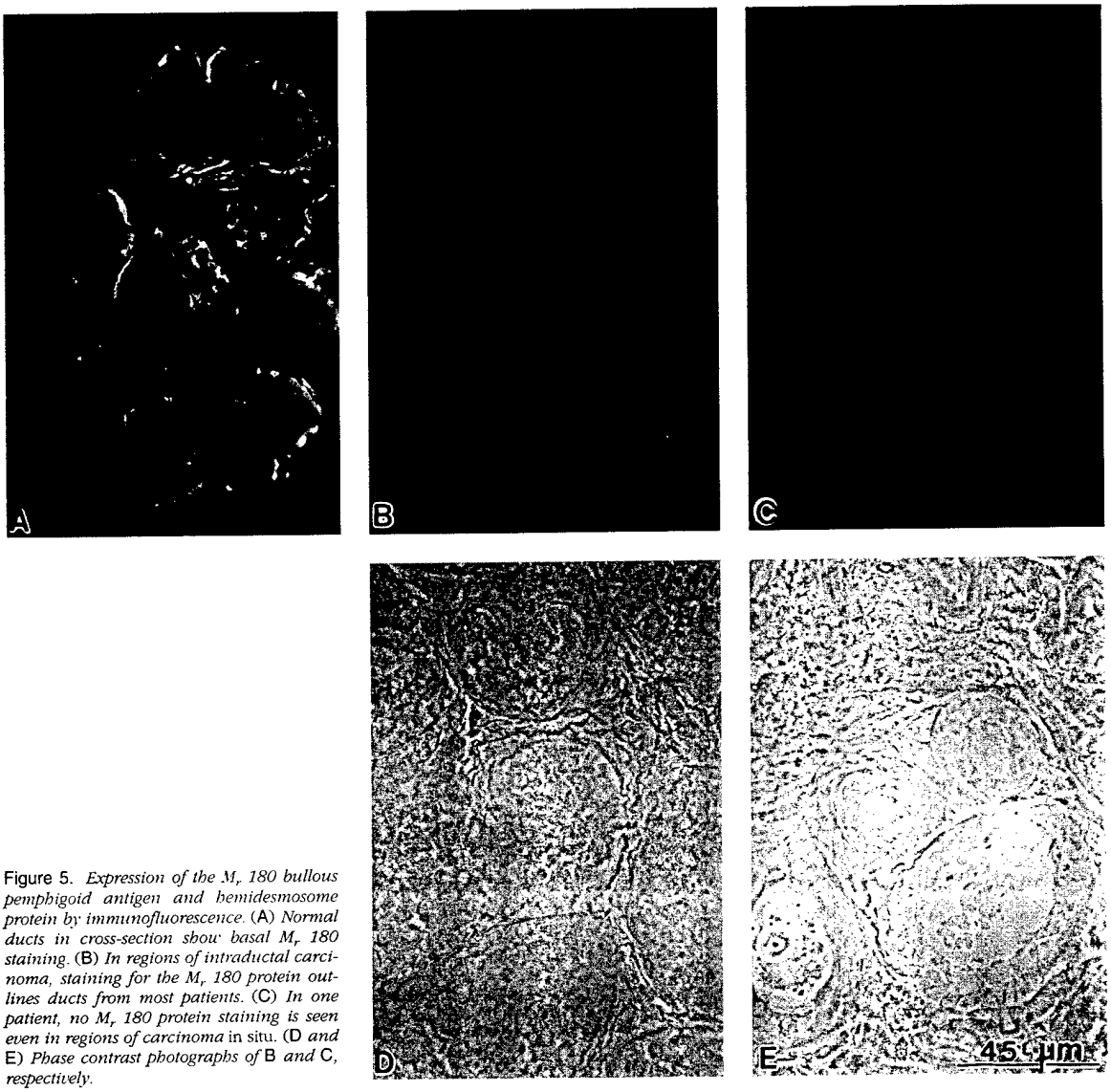

Figure 5. *Expression of the M_r 180 bullous pemphigoid antigen and hemidesmosome protein by immunofluorescence.* (A) *Normal ducts in cross-section show basal M_r 180 staining.* (B) *In regions of intraductal carcinoma, staining for the M_r 180 protein outlines ducts from most patients.* (C) *In one patient, no M_r 180 protein staining is seen even in regions of carcinoma* in situ. (D *and* E) *Phase contrast photographs of* B *and* C, *respectively.*

cytosolic pattern at incubations as long as 30 days (Figure 9). The protein was never expressed basally by malignant cells. The M_r 230 and 180 proteins exhibited lines of basal tick-marks in malignant cells just as in normal cells and with the same time course (Figure 10). As reported previously, the β4 integrin subunit was also expressed identically in normal and malignant cells, whereas the α6 subunit was seen in only ≈30% of malignant cells in culture.[5]

Hemidesmosome Assembly and Regulation

Because normal HMEC formed HD in culture, we used them as a model to study HD assembly. In time courses of HD protein expression as determined by IF, all but one protein were seen intracellularly within 24 hours of plating, but a mature basal plaque-like distribution of proteins was seen with only α6 and the M_r 180 protein (Table 1). Other proteins achieved their mature localization in a specific order (Table 1). HD were seen at the EM level for the first time at 2 weeks.

Because HMEC are highly mobile cells at low cell densities, and HD are structures involved in stable attachment, we wondered whether assembly might be affected by cell density. In fact, localization of the M_r 230 and 200 proteins, β4, and collagen VII were highly dependent on confluence (Table 2). At 14 days in the majority of cells studied when they were at 25% confluence or less, the M_r

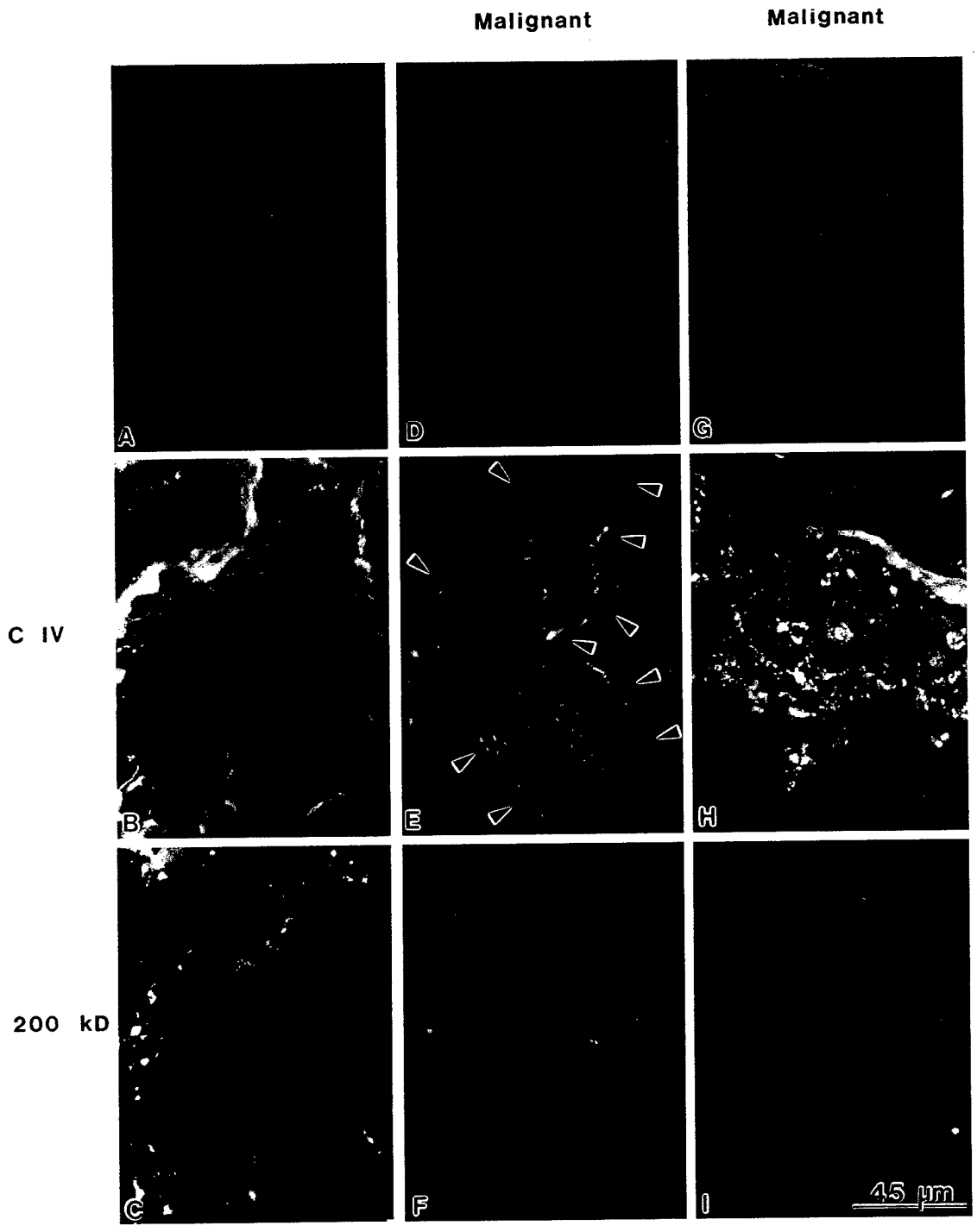

Figure 6. *Expression of the M_r 200 HD protein does not follow the BM by immunofluorescence.* (A) *Negative control for immunofluorescent staining.* (B, C) *A normal duct in cross section has a BM illustrated by collagen IV staining* (B) *and expresses basolateral M_r 200 protein* (C). (D–F) *An intraductal carcinoma in longitudinal section* (D) *has a basement membrane* (E, arrowheads), *but no M_r 200 protein staining* (F). (G–I) *Invasive carcinoma* (G) *exhibits intracellular BM protein staining* (H), *and faint M_r 200 protein staining surrounding each cell* (I). *C IV, collagen IV.*

230 and 200 proteins and collagen VII were localized intracellularly, and β4 was not present, whereas at >40% confluence the four proteins were all present and basally located. On the other hand, α6 and M_r 180 protein localization were insensitive to cell density; these proteins were basally located in all cells at 14 days regardless of confluence.

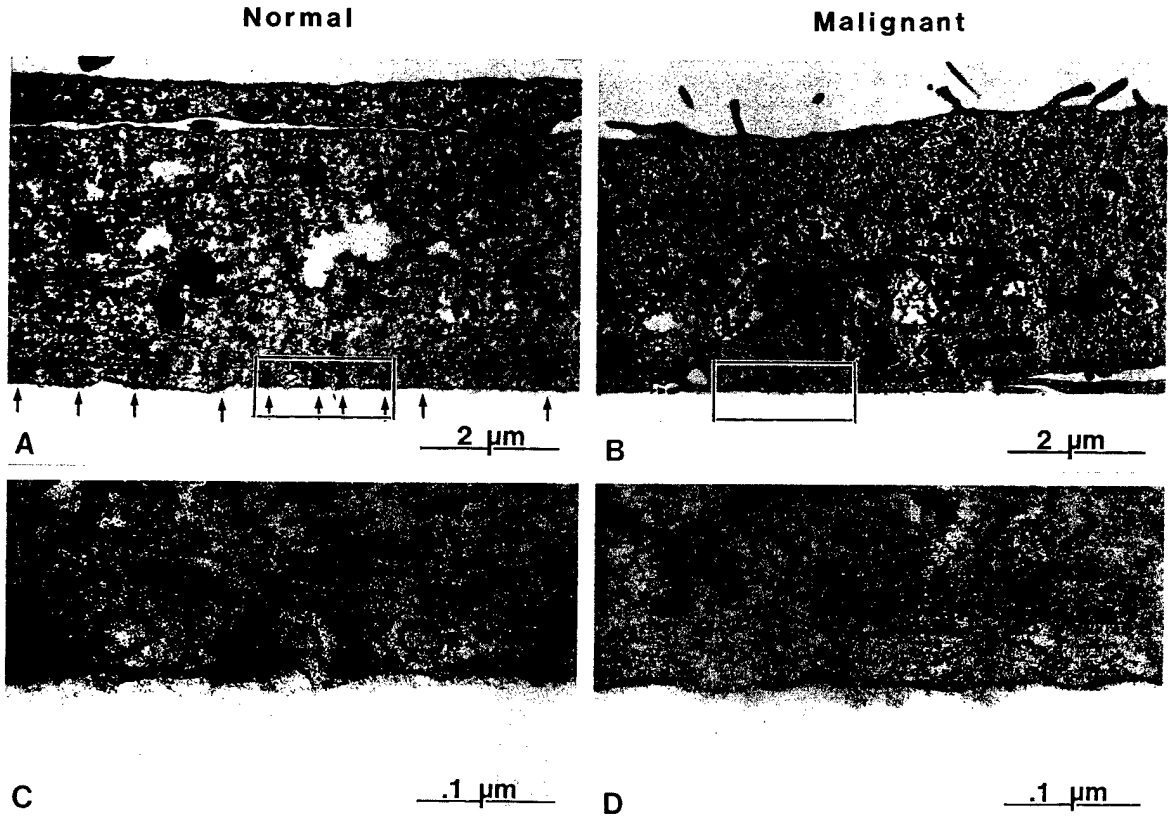

Figure 7. *Electron microscopy of normal and malignant HMEC in culture.* (A) *A normal cell has basal HD* (arrows). (B) *A malignant cell has no HD.* (C) *Higher magnification of box in* A. (D) *Higher magnification of box in* B.

Cell proliferation also influenced HD assembly. We compared protein expression between three cell strains with rapid doubling rates and three cell strains with slow doubling rates by plating cell concentrations that would result in >50% confluence at 2 weeks. Rapidly proliferating cells did not localize the M_r 230 and 200 proteins, β4, or collagen VII basally after the usual 2 weeks, whereas slower growing cells did (Table 3). α6 and M_r 180 protein localization were independent of doubling time as they were of cell density. Even rapidly proliferating cells promptly localized all expected proteins when they reached confluence.

Because motility and proliferation, which appeared to delay HD protein expression, can reflect a dedifferentiated phenotype, we wondered if differentiation might conversely stimulate HD assembly. We plated normal HMEC on Matrigel, a BM-like substance that promotes HMEC morphogenesis and functional differentiation.[30, 38] Electron-dense HD were seen by EM at the bases of cells in fully formed three-dimensional ductlike structures (Figure 11), but not in cells on Matrigel that were still migrating to form these structures (not shown). The process of duct formation was complete at between 14 and 24 days. Likewise, HD were first seen between 14 and 24 days whenever duct formation was complete. Thus HD formation on matrigel appeared to be differentiation-dependent rather than time-dependent.

Finally, because expression of HD in malignant cells *in vivo* appeared to correlate with the presence of BM proteins, we wondered whether ECM might actually stimulate HD assembly. For this purpose we used the laminin-5-rich matrix produced by 804G cells and previously shown to induce rapid HD formation in skin cells.[31] Normal HMEC plated on this matrix formed electron-dense HD within 24 hours instead of the usual 2 weeks (Figure 12). This ECM does not promote HMEC morphogenesis.

Discussion

In previous detailed EM studies of the breast, HD were noted at the BM in basal cells.[23–28] This information seems to have gone relatively unnoticed in the HD literature, where HD continued largely to be discussed as characteristic of stratified epithelia. In addition, it was not clear from past studies whether HD were unique to breast myoepithelial cells or used by all breast epithelial cells to attach to the BM.

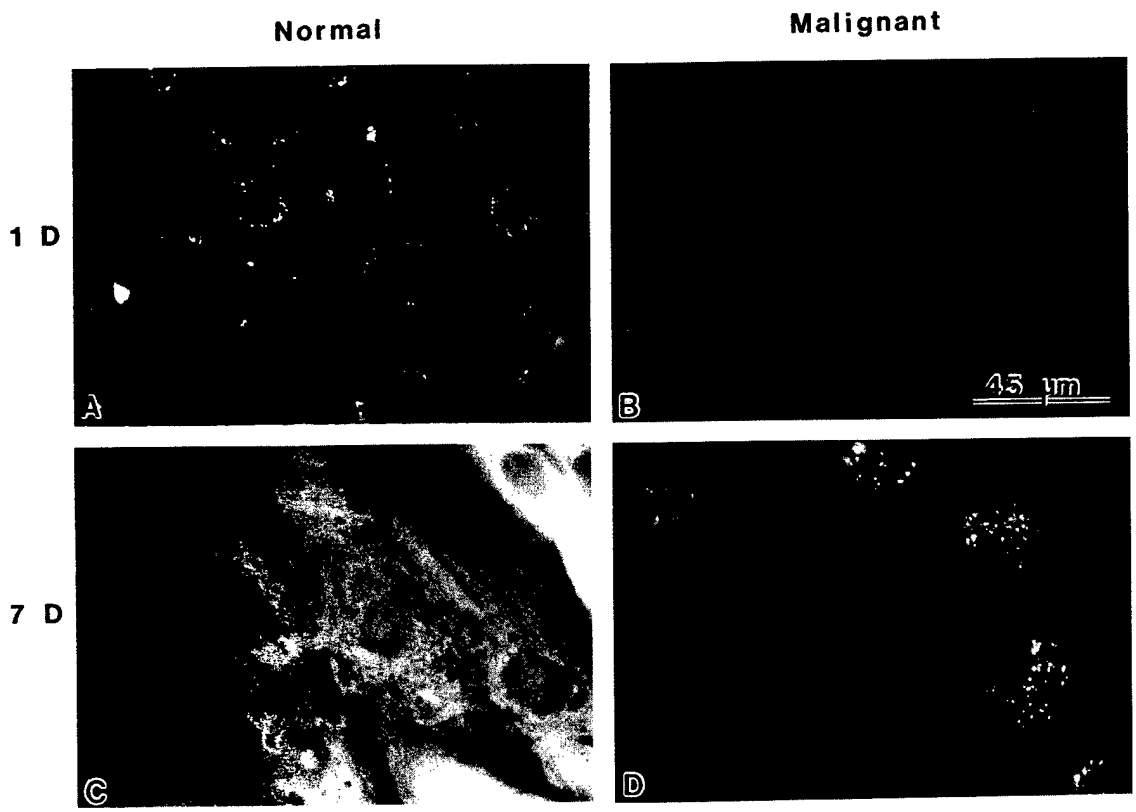

Figure 8. *Delayed expression of the anchoring fibril protein, collagen VII, in cultured malignant HMEC by IF.* (A) *Normal cultured HMEC at 1 day show an intracellular staining pattern for collagen VII.* (B) *Malignant HMEC do not express collagen VII at 1 day.* (C) *By 7 days normal cells stain for collagen VII at the base of cells in a secreted pattern.* (D) *At 7 days malignant cells show an intracellular staining pattern for collagen VII.*

In this study we clearly demonstrate that HD are found where all breast cells, whether myoepithelial or luminal, are in apposition to the BM. This strengthens the data adding breast epithelial cells to the expanding list of cell types that express HD, which now includes stratified epithelia such as skin, cornea, and esophagus[10, 39]; complex epithelia such as trachea, thymus, and transitional epithelia of the urinary bladder[10]; glandular epithelia such as apocrine and salivary glands[26, 10]; and even the simple epithelium of the amnion.[40]

We would like to emphasize that we noted that luminal cells as well as myoepithelial cells contained HD. Because breast carcinoma cells tend to differentiate toward a luminal phenotype in, eg, expression of cytokeratins and actins, it is important to note that absence of HD from invading breast carcinoma cells does not merely represent loss of surrounding myoepithelial cells, but a clear downregulation of HD expression. Recently Clermont et al[41] studied collagen VII anchoring fibrils in the rat breast and also emphasized that both luminal and myoepithelial cells contain abundant HD *in vivo*.

We also determined that normal breast epithelium expressed at least six of the known HD protein components, suggesting that HD have a similar structure and play a similar role in breast as they do in skin and other stratified tissues.

HD are probably involved more in stable rather than motile adhesion. For example, $\alpha 6\beta 4$ was found only in nonmotile keratinocytes in culture,[15] and HD are downregulated in epithelial cells that become motile to fill in a wound (see, eg, refs. 36, 42).

That HD are in fact adhesive structures used by normal epithelia to adhere to BM is suggested by wound healing studies in which reepithelialized cornea can be easily lifted off until BM and HD have formed.[43] Further, the epithelium releases the stroma as a sheet in the genetic blistering diseases dystrophic epidermolysis bullosa, in which anchoring fibrils are congenitally absent,[44, 45] and lethal junctional epidermolysis bullosa, in which HD are abnormal[46]; and in the acquired diseases epidermolysis bullosa aquisita and bullous pemphigoid, in which autoantibodies to collagen VII and to the M_r 230 and 180 HD components, respectively, are found.[47, 48] Also, experimental addition of blocking antibodies to HD proteins either *in vivo* or in culture causes loss of epithelial adhesion.[42]

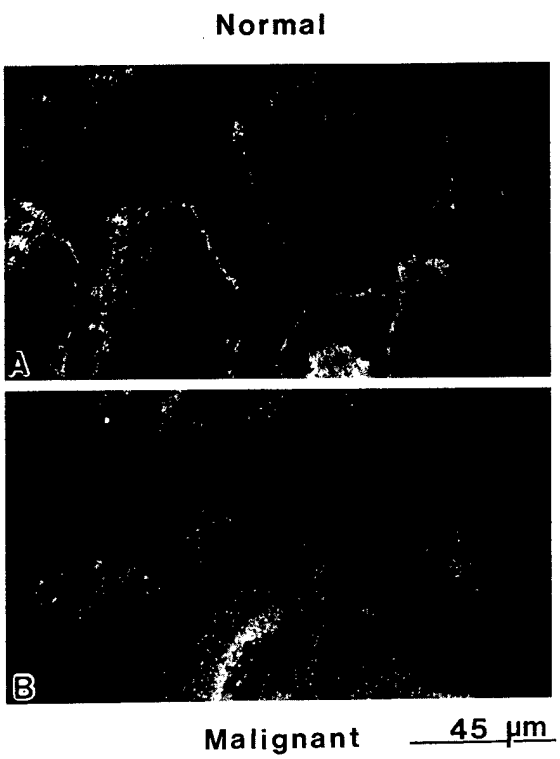

Figure 9. *Aberrant expression of the M_r 200 HD protein in cultured malignant HMEC by IF.* (A) *Normal cells express the M_r 200 protein in rows of basal tick-mark-shaped plaques.* (B) *Malignant cells show a dotted intracellular M_r 200 protein staining pattern.*

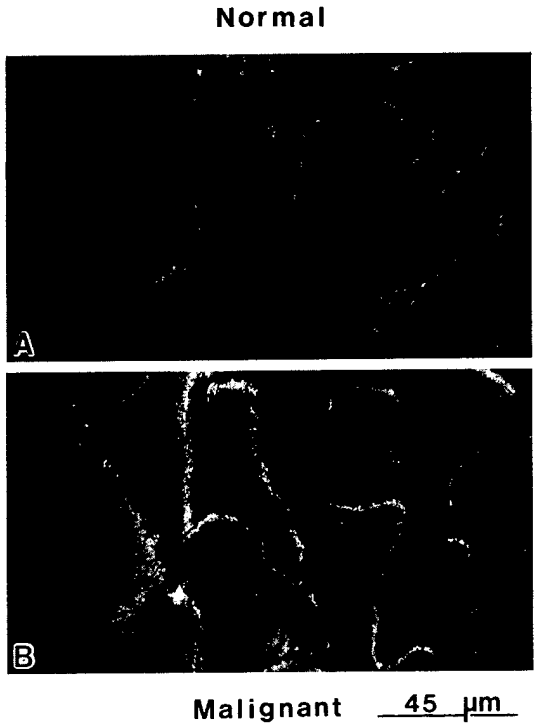

Figure 10. *Identical expression of the M_r 180 HD protein in normal and malignant cultured HMEC. Both normal* (A) *and malignant* (B) *cells express the M_r 180 protein in rows of basal tick-mark-shaped plaques.*

The lack of HD in invasive breast carcinoma seen in this study may allow such cells to become less adherent to the BM. During branching morphogenesis that occurs in the breast during embryogenesis, cells in the penetrating endbud lose expression of HD when they invade the stroma and branch into new ducts.[27, 41]

Interestingly, all tumors downregulated the same HD proteins at the same locations regardless of their stage or grade. There are two possible reasons for this. 1) Certain steps in dedifferentiation may typically occur in the same pattern. This apparent pattern relates to the normal pattern of assembly we saw *in vitro*. The proteins that assembled early (M_r 180, α6, collagen VII, and also M_r 230) appear to be coregulated in carcinoma with BM, and may be the proteins that receive a signal from the BM to nucleate HD formation in the normal breast. In fact, α6 and M_r 180 are transmembrane proteins that probably play a role as ECM receptors. On the other hand, M_r 200 and β4 were lost in carcinoma independent of BM and were among the last to be expressed in normal HD formation. Additionally, it was the late proteins that were apparently dependent on motility and proliferative rate for their assembly into HD. 2) Our sample size and exclusive use of ductal carcinoma may have biased our results; and it would be interesting in future studies to compare tumors of different types (e.g., lobular, mucinous, ductal), stages, and grades in sample size with sufficient statistical power to detect differences between subgroups.

Another interesting observation in this study regards HD expression in intraductal carcinoma *in vivo*. We noted HD in the basal cells by EM; however, by IF there were many regions in which the M_r 200 protein and β4 integrin were not expressed by basal cells. This suggests that even in intraductal carcinoma, the apparently ultrastructurally normal HD may actually be abnormal, and could be functionally impaired. Cells piled up within ducts and away from the BM in carcinoma *in situ* had no HD and lacked staining for component proteins. Abnormal downregulation of HD may allow these malignant cells to leave the BM. Alternately, if the BM regulates HD expression, cells that have left the BM with HD intact may then downregulate HD expression as the normal response to a loss of contact with the BM. This brings up the cause and effect question of HD and BM expression: does the BM regulate HD expression or vice versa?

Our *in vitro* results suggest that ECM stimulates HD formation in HMEC. HD formation was accelerated from the 14 days seen on glass to one day by

Table 1. *Time Course of Expression and Localization of HD Proteins and Electron-Dense HD in Normal HMEC in Culture*

	180 kd	α_6	Collagen VII	230 kd	200 kd	β_4	HD visible by EM
Intracellular localization (days after plating)	–*	–	1	1	1	–	–
Mature basal localization (days after plating)	1	1	5–7	10	10	14	14

Average of three experiments using four cell strains. Cells were >50% confluent and of average doubling time. Please refer to text for description of HD proteins.

* = Lack of intracellular localization, these proteins were found at their mature basal location without apparent previous intracellular localization.

Table 2. *Dependence of Basal Localization of HD Proteins in Normal HMEC in Culture on Cell Density*

Plating density (no. cells/35 mm^2)	Final confluence (visual estimates at 14 days)	% of cells/well with basal distribution of 230 kd, 200 kd, collagen VII and β_4	% of cells/well with basal distribution of 180 kd and α_6
5×10^3	5–10%	5 ± 5	100
10^4	15–25%	45 ± 15	100
5×10^4	40–75%	100	100

Average of two experiments using three cell strains. Numbers are mean ± SD. Cells were of average doubling time.

culture on 804G cell matrix. Although a similar effect of matrix on HD assembly was previously noted when epithelial cell lines that did not form HD on plastic were able to make them on collagen I, the impact of 804G matrix is much more rapid.[50, 51, 52] In addition, in embryogenesis, expression of BM clearly precedes HD expression (see, e.g., refs. 53–55). However, most wound healing studies show that in epithelial cells that have migrated to fill a wound, HD reform before or simultaneously with BM.[36, 42, 56–61] In fact, BM initially reforms discontinuously beneath HD as if the HD nucleate BM formation.[44, 62, 63]

It is not clear which of these models best reflects the situation in the normal breast or in breast cancer. In our study there appeared to be a correlation between localization of a cell at the BM and its expression of HD. Therefore the BM could be regulating HD expression. However, some cells at the BM did not express every HD protein, and some cells away from the BM did express some HD proteins. We therefore believe that although lack of HD in invasive breast cancer could be due to a loss of BM, some HD proteins are probably downregulated independently of the BM. In addition, Wetzels et al[64] found, as we did, that collagen VII was lost from almost all (94 of 97) invasive ductal carcinomas, but 13 of these retained staining for BM proteins, suggesting that loss of this HD protein was not a result of loss of BM protein.

It is also possible that the invasive phenotype is an expression of dedifferentiation in general such that both HD and BM protein expression are downregulated by a preceding dedifferentiation event. Such dedifferentiation is seen in wound healing when ep-

Table 3. *Dependence of Basal Localization of HD Proteins in Normal HMEC in Culture on Rate of Proliferation*

Doubling time (days)	% of cells with basal localization of 230 kd, 200 kd, collagen VII, and β_4 at 2 weeks	% of cells with basal localization of 180 kd and α_6 at 2 weeks
3	45 ± 15	100
10	95 ± 5	100

Three cell strains with shorter doubling times (3 days) were compared with three cell strains with longer doubling times (10 days) in three experiments. Numbers are mean ± SD.

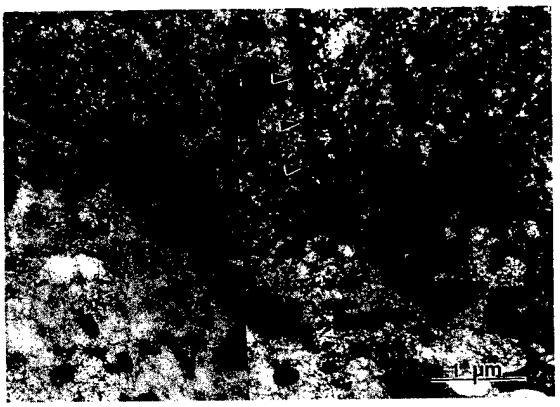

Figure 11. *EM of normal HMEC cultured on Matrigel. Cells were grown on Matrigel BM-like substance for 20 days. During this time they underwent differentiation into three-dimensional ductlike structures and assembled HD* (arrows), *which were associated with intermediate filaments* (arrowheads).

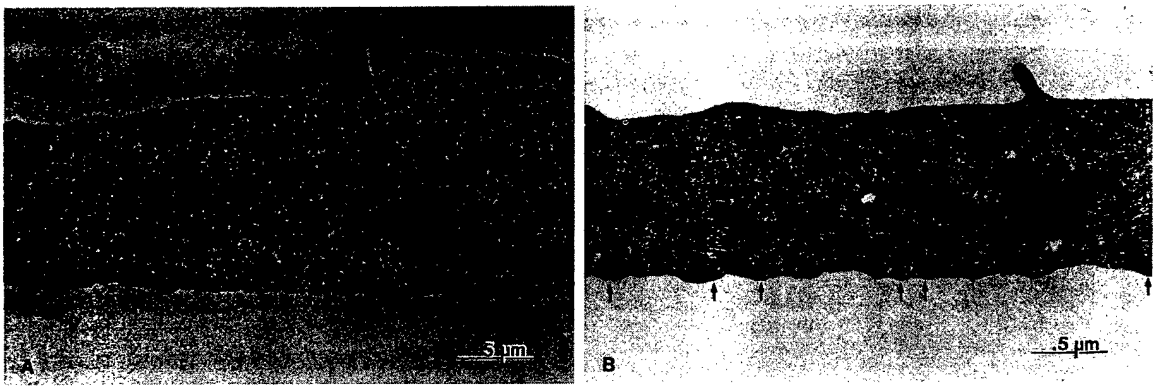

Figure 12. *EM of normal HMEC cultured on 804G matrix.* (A) *HMEC grown on glass coverslips for 24 hours have no HD.* (B) *HMEC grown on laminin-rich 804G matrix for 24 hours contain HD* (arrowheads).

ithelial cells migrate over stroma to reepithelialize denuded regions, and HD and BM proteins are decreased in expression[57, 54] or at least relocated.[42, 65] In addition, HD formation appears to be linked to differentiation events in HMEC, as normal cells on Matrigel formed HD in a manner temporally linked with the differentiation that occurred on that matrix.

With respect to our observations on HD formation in culture, most groups that have reported previously on cells cultured on plastic or glass have seen either no HD by EM, or "prehemidesmosomes" or "immature hemidesmosomes" (reviewed in refs. 11, 65, 66). Recently, however, a few rat and bovine cell lines able to make mature HD have been reported (see, e.g., refs. 10, 66, 67). In this paper we report that our normal primary human cells form HD in culture containing the anticipated protein components. This suggested these breast epithelial cell strains as good models for the study of HD formation.

In time course studies of HD protein expression, most HD proteins were already being made as soon as cells were attached enough to be stained, but they were not all basally located into mature plaques, and EM-recognizable HD were not made until 2 weeks in culture. The order of assembly into mature plaques appears to be α6 and M_r 180 at day 1, then collagen VII, M_r 200 and 230, and finally β4 at the time of final assembly. Kurpakus et al[35, 36, 42] have outlined the order of HD protein assembly in a wound healing tissue culture model, and they also see early appearance of α6, but accompanied by β4. Other HD proteins assemble in a somewhat variable order.[35, 36, 42] It is somewhat surprising that α6 was expressed before β4 in our model, as these two integrin subunits pair to form one integrin molecule in HD. However, the α6 subunit has two possible partners, β1 and β4, to form two different integrins. We see expression of the β1 subunit simultaneous to α6 on day one,[5] but colocalization by IF of α6 and β4 by day 14.[5]

Except for the first two proteins to be localized basally, α6 and M_r 180, the HD proteins studied remained intracellular while cells were mobile or highly proliferative. This suggests that these cells are able to couple HD localization to machineries of cell cycle and motility. This is consistent with data in the skin in which epithelial cells proliferating and migrating to cover a wound contain HD proteins intracellularly only, but assemble basal HD when migration and proliferation are complete.

Several of the parameters that appear to regulate HD expression by normal HMEC are factors perturbed in carcinoma: motility, proliferative rate, and BM synthesis. In fact, these interrelationships may play a role in the downregulation of HD we observed in malignancy. Several malignant breast cell strains in culture lacked HD by EM; however, some malignant cells in culture did express HD. This heterogeneity probably reflects the heterogeneity of the tissues from which the cells were derived. For example, in cells grown from one tumor, some cells were seen with and others without HD by EM; these cells could represent intraductal and invasive cells, respectively. In the one cell strain where we saw a normal complement of HD, only intraductal cells may have grown; and in the three cell strains with no HD, only invasive cells may be represented. It is also possible that cells derived from higher stage or grade tumors might exhibit the more abnormal phenotype. This could not be determined from the sample size in this study, but warrants further investigation.

Malignant cells in culture had a more normal array of HD protein expression by IF than did malignant cells *in vivo*. As mentioned above, it is possible that the cells grown in culture were not fully representative of those found *in vivo*. For example, if a majority

of the cells in culture were derived from the intraductal portion of carcinomas, a more normal phenotype would be observed.

A more interesting possibility is that HD proteins not expressed *in vivo* can be reexpressed by tumor cells in a different milieu in culture. This suggests that the ability to express HD proteins may not be lost by malignant cells; rather, HD proteins may be downregulated *in vivo* by a program of altered differentiation that is partially reexpressed in culture. This was also suggested by the fact that malignant cells *in vivo* had such a complete change in HD protein expression, when a loss of only one protein might have been expected if the loss were due to a mutation in that gene. It is further supported by the fact that collagen VII, which is eventually expressed basally in malignant cells in culture as in normal cells, exhibits a delayed conversion to the normal phenotype from that of intracellular expression. However, the fact that the M_r 200 protein remained abnormal in culture suggests the possibility of a permanent change in expression of this particular protein. Therefore, both the regulation of HD proteins and some of the proteins themselves may be abnormal in breast cancer. This hypothesis will have to be tested further in subsequent studies.

As mentioned above, malignant breast cells in culture did exhibit some abnormalities of HD protein expression by IF: the M_r 200 protein and collagen VII were seen intracellularly. This localization is reminiscent of wound healing, in which HD proteins are also expressed intracellularly by epithelial cells migrating to close a wound.[68] Some aspects of cancer invasion have been compared with wound healing, and internalization of HD components could be a common mechanism by which epithelial cells become migratory.

In conclusion, in this paper we note the presence of HD in normal HMEC both *in vivo* and in culture containing the expected proteins, and a correlation of increasing downregulation of expression of HD with increasing aggressiveness of tumor cells. Such a correlation has been previously noted in other malignancies and with other adhesion molecules (reviewed in refs. 69–71; see also refs. 72, 73 for recent updates). We expand their data to include HD and breast cancer, and suggest that normal HMEC may use HD to maintain their position in the mammary duct, and malignant cells may use downregulation of HD as a means of escape from usual tissue architectural restraints.

Further, our manipulations of cells in culture together with *in vivo* data suggest a model for HD protein regulation. Early HD protein localization may be signaled by the extracellular matrix and regulated by it, whereas localization of late HD proteins (those usually localized to cell bases later) appear to be coupled to motility and proliferation. Both sets of proteins are affected in malignancy in which ECM, motility, and proliferation are all abnormal. Because HD expression appears to be linked to differentiation, this may reflect a program of dedifferentiation by malignant cells *in vivo* that may be able to be partially reversed under certain circumstances (e.g., in culture) suggesting that these malignant changes may be a combination of genetic and epigenetic events.

Acknowledgments

We gratefully acknowledge Rob Mihalick and the Electron Microscopy Facility for technical assistance, Dr. Daniel Snower for pathology consultation, Dr. Kathy Green for help with immunofluorescence, and Marie Lee and Zarah Castillo for manuscript preparation.

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